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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. Prior to 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 multi-species 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.

Key words: titi monkeys, New World monkeys, phylogeny, taxonomy

Introduction

The titi monkeys are small to medium sized (adult body weight 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 had 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 sample 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 et al. (2002), who also considered all the 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 et al. (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 compiled the *torquatus* species group of Hershkovitz (1988, 1990), Groves' (2005) *Torquatus* subgenus, 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 the *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, however, due primarily to the sampling deficiencies of many areas, as in the case of *C. lucifer* and *C. medemi*, which both 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 *et al*. (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 phylogenetic arrangement of the species of the genus based on DNA sequencing of mitochondrial and nuclear markers.

Material and Methods

Samples, and the 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 (1 *C. torquatus*, 6 *C. lugens*, 3 *C. purinus*, 3 *C. lucifer*, 4 *C. regulus*, 3 *Plecturocebus*, 3 *Callicebus*, 1 *Chiropotes*, 1 *Cacajao*, and 1 *Pithecia*). No samples of *Cheracebus medemi* could be obtained for analysis in the present study. The samples (Table 2, 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 Manaus, 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.

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 (1.25mM); 1.5 µl of 10X 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 95°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 extension at 72°C for 5 min. The PCR products were purified with polyethylene glycol (PEG) and ethanol. The sequence reactions were run with the Big Dye kit (Applied Biosystems), and the samples were sequenced in an ABI 3500 XL automatic sequencer (Applied Biosystems). The access numbers on GenBank of the sequences produced in the present study are available in the supplementary table S1.

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

The DNA sequences were aligned in ClustalW (Thompson *et al*., 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 *et al*., 2016) was used to identify the best data partitioning scheme and evolutionary models. We used the greedy algorithm (Lanfear *et al*. 2012) and the Bayesian Information Criterion (BIC) and protein coding regions were partitioned by position of the bases in the codons. Were performed analysis 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 the supplementary files (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 was found by 1000 searches followed by 1000 bootstrap pseudoreplicates. The BI was run in MrBayes v.3.2.1 (Ronquist and 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 5000 generations. The first 20% of the runs were discarded as burn-in. The species tree with a multi-species coalescent model was estimated with ASTRAL III (Zhang *et al*., 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 *et al*. 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 *et al*., 2012), using two calibration points: (i) the *Cacajao–Chiropotes* separation, estimated at 6.7±2.3 million years ago (Ma) (Kiesling et al. 2015); (ii) 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 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 *et al*., 2014), and an Effective Sample Size (ESS) of over 200 was considered to be satisfactory.

Results

The 16 concatenated markers (nuclear and mitochondrial) provided a database of 9427 base pairs (bps), 2181 bps from the mitochondrial sequences, and 7246 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 allelic diversity within species was reciprocally monophyletic, and all the relationships within the genus *Cheracebus* were strongly supported. The Phylogenetic analysis under the multi-species coalescent model (Figure 3) recovered the same topology of probabilistic methods (ML and IB), also with most of the nodes strongly supported. We obtained incongruity in the phylogenetic position of *C. torquatus* when analyzed the mitochondrial and nuclear data separately (Figura S1). Only mitochondrial data groups *C. torquatus* within of *C.*

lugens, with 60% of bootstrap, making paraphyletic *C. lugens*. 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 has an average genetic distance 5.17 times greater than nuclear data (Table 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).

Discussion

Until recently, the titi monkeys were classified in 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 recuperated as monophyletic groups in the present analysis, which is consistent eith the 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 determined 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.

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 were 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 ample 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.

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 reciprocal, monophyletic clades 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*.

Ethics

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 N° 005/2005 – CGFAU/LIC) and ICMBio (40217-1 and 5135-1).

Competing interests

We have no competing interests

Authors' contributions

JC conceived of the study, participated in the data analyses and drafted the manuscript; IS designed the study, provided samples; TL carried out the molecular laboratory work and drafted the manuscript, JSSJ provided input on the manuscript, and revised the text; JB, IF, TH and JV provided samples and revised the manuscript; HS 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.

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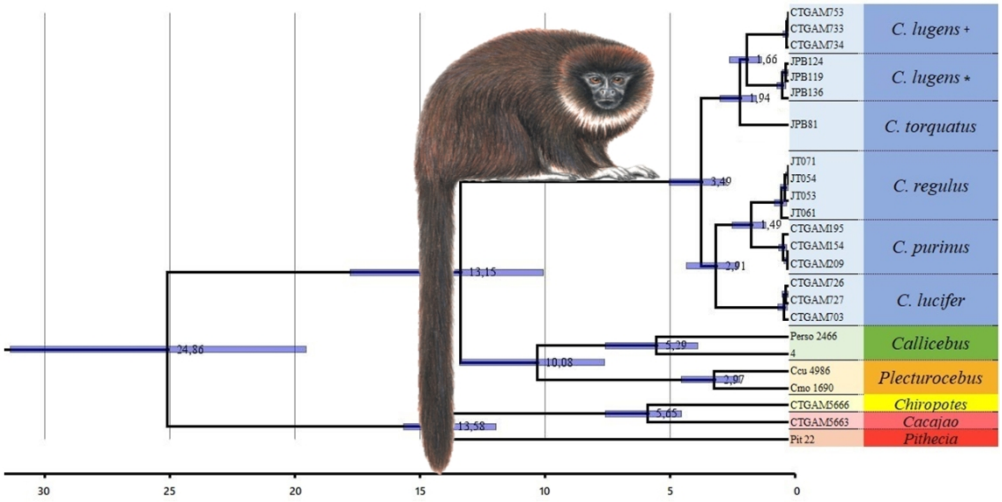
Zang, C., Rabiee, M., Sayyari, E., & Mirarab, S. (2018). ASTRAL-III: polynomial time species tree reconstruction from partially resolved gene trees. *BMC bioinformatics*, 19(6), 153. https://doi.org/10.1186/s12859-018-2129-y.



338x190mm (96 x 96 DPI)

Highlights:

- *Cheracebus* is a genus of the subfamily Callicebinae;
- *Cheracebus* lineages originated approximately 13 ma ago;
- The phylogenetic relationships between the species of th genus *Cheracebus* are as follows: ((*C. torquatus*, *C. lugens),* (*C. lucifer*, (*C. purinus*, *C. regulus*))).

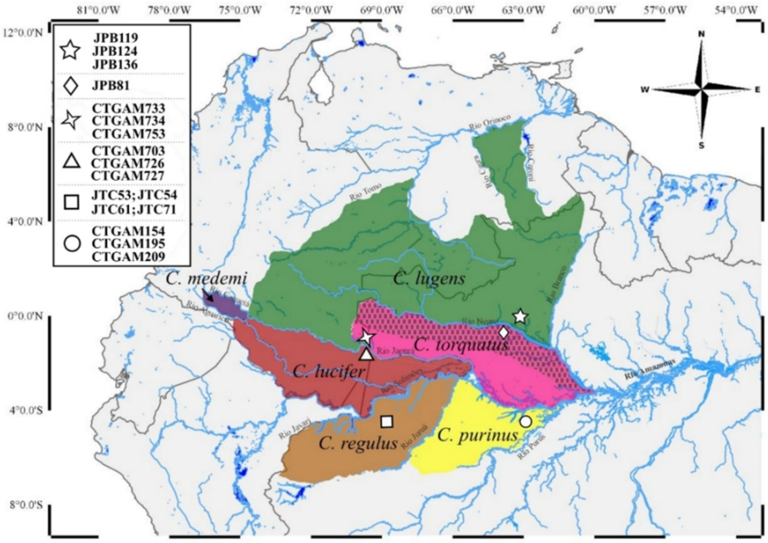


Figure 1. Distribution map of *Cheracebus* species (Hershkovitz, 1990; van Roosmalen et al., 2002). Dotted region represents a possible zone of sympathy between *C. lugens* and *C. torquatus* species. The symbols represent the locations where the samples were collected.

338x190mm (96 x 96 DPI)

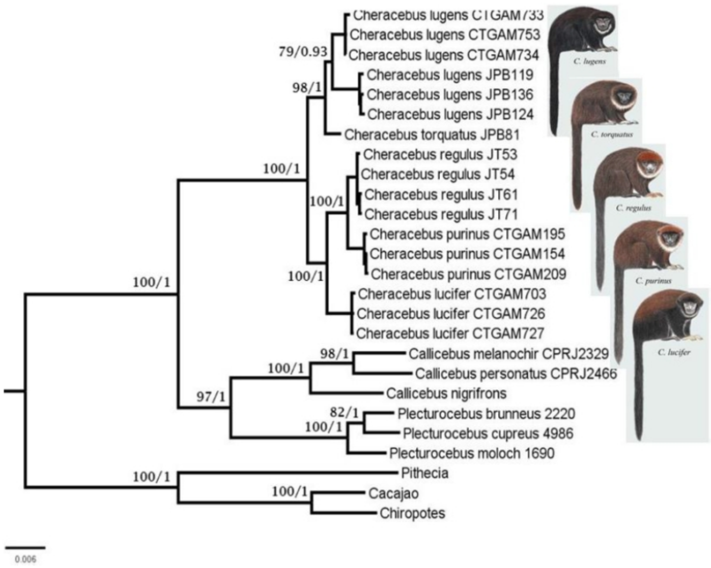
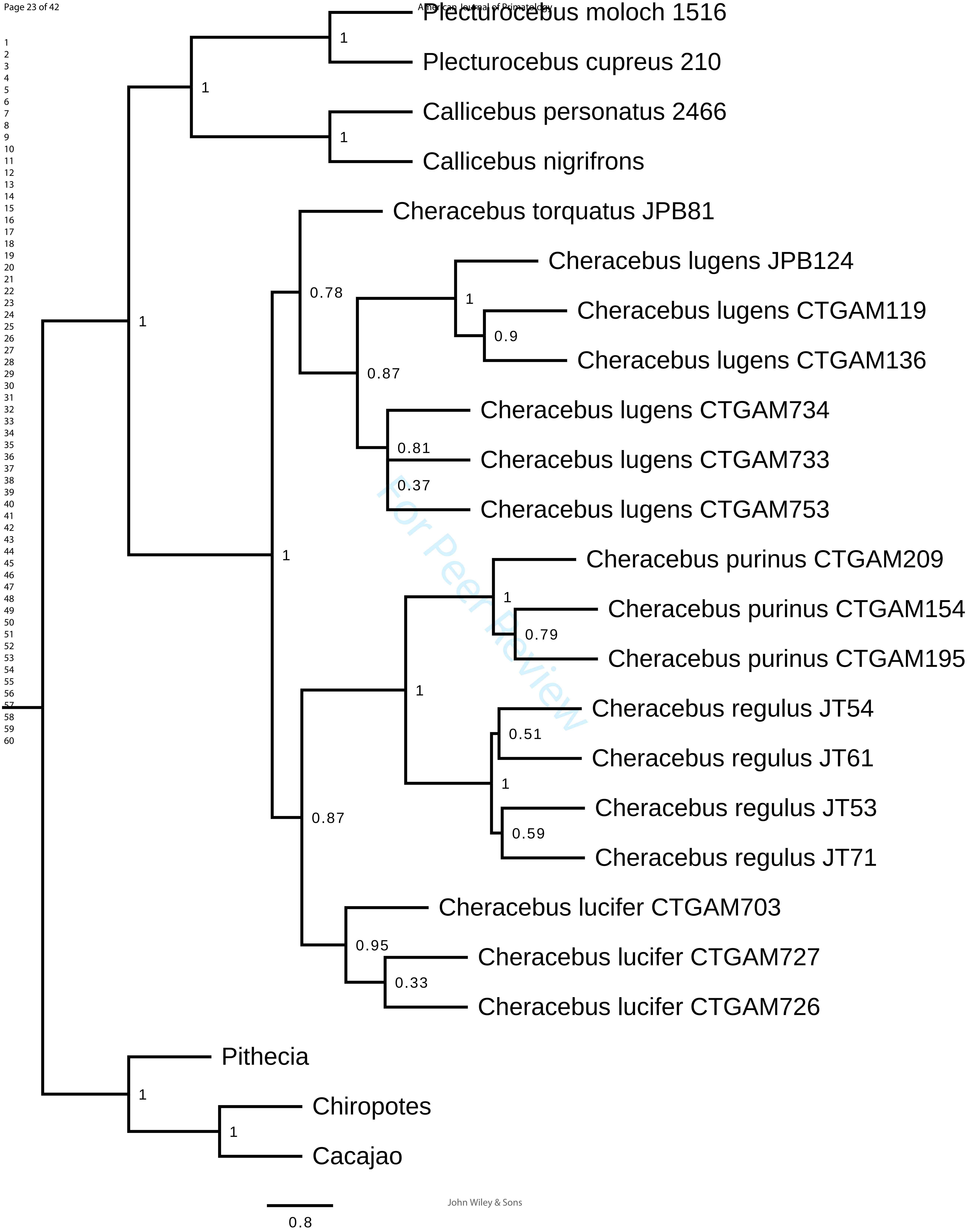


Figure 2. Phylogenetic relationships between taxa of the Pitheciidae family. Numbers near nodes refer to bootstrap (left) and posterior probability (right) values.

338x190mm (96 x 96 DPI)


Plecturocebus moloch 1516
1
Plecturocebus cupreus 210
1
Callicebus personatus 2466
1
Callicebus nigrifrons
Cheracebus torquatus JPB81
Cheracebus lugens JPB124
0.78
1
Cheracebus lugens CTGAM119
1
0.9
Cheracebus lugens CTGAM136
0.87
Cheracebus lugens CTGAM734
0.81
Cheracebus lugens CTGAM733
0.37
Cheracebus lugens CTGAM753
1
Cheracebus purinus CTGAM209
1
Cheracebus purinus CTGAM154
0.79
Cheracebus purinus CTGAM195
1
Cheracebus regulus JT54
0.51
Cheracebus regulus JT61
1
Cheracebus regulus JT53
0.87
0.59
Cheracebus regulus JT71
Cheracebus lucifer CTGAM703
0.95
Cheracebus lucifer CTGAM727
0.33
Cheracebus lucifer CTGAM726
Pithecia
1
Chiropotes
1
Cacajao
0.8

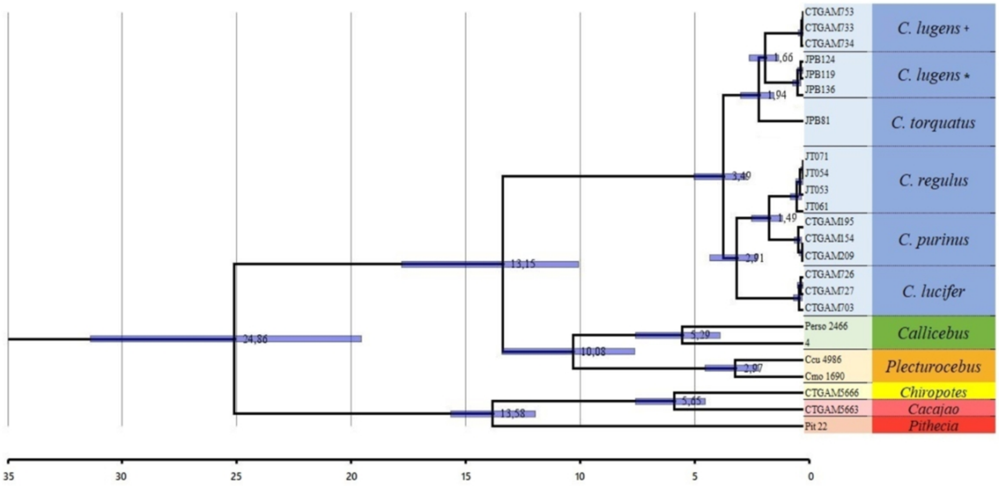


Figure 4. Estimated divergence time between Pitheciidae taxa. Each genus has a color: blue to Cheracebus, *green to* Callicebus, *orange to* Plecturocebus, *yellow to* Chiropotes; *pink to* Cacajao *and red to* Pithecia. *(*) highlights clade of* C. lugens *on the left bank of the river Negro, while (+) indicates the samples collected on the right bank of this river. Numbers next node represent the average time estimated by cladogenesis*

338x190mm (96 x 96 DPI)

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*
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*

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 river Negro, Amazonas, Brazil
Cheracebus lugens	JPB119	INPA	Marari, left bank of river Negro, Amazonas, Brazil
Cheracebus lugens	JPB124	INPA	Igarapé Anta, left bank of river Negro, Amazonas, Brazil
Cheracebus lugens	JPB136	INPA	Igarapé Cuieiras, left bank of river Negro, Amazonas, Brazil
Cheracebus lugens	CTGAM733	UFAM	Left bank of river Japurá, Amazonas, Brazil
Cheracebus lugens	CTGAM734	UFAM	Left bank of river Rio Japurá, Amazonas, Brazil
Cheracebus lugens	CTGAM753	UFAM	Left bank of river Japurá, Amazonas, Brazil
Cheracebus purinus	CTGAM154	UFAM	Rebio Abufari, left bank of river Purus, Amazonas, Brazil
Cheracebus purinus	CTGAM195	UFAM	Rebio Abufari, left bank of river Purus, Amazonas, Brazil
Cheracebus purinus	CTGAM209	UFAM	Rebio Abufari, left bank of river Purus, Amazonas, Brazil
Cheracebus lucifer	CTGAM703	UFAM	Right bank of river Rio Japurá, Amazonas, Brazil
Cheracebus lucifer	CTGAM726	UFAM	Right bank of river Rio Japurá, Amazonas, Brazil
Cheracebus lucifer	CTGAM727	UFAM	Right bank of river Rio Japurá, Amazonas, Brazil
Cheracebus regulus	JT053	UFPA	Right bank of river Jutaí, Amazonas, Brazil
Cheracebus regulus	JT054	UFPA	Right bank of river Jutaí, Amazonas, Brazil
Cheracebus regulus	JT061	UFPA	Right bank of river Jutaí, Amazonas, Brazil
Cheracebus regulus	JT071	UFPA	Right bank of river Jutaí, Amazonas, Brazil
Plecturocebus moloch	Cmo 1690	UFPA	Left bank of river Tocantins, Amazonas, Brazil
Plecturocebus brunneus	Cbr 2220	UFPA	Right bank of river Jamari, Rondonia, Brazil
Plecturocebus cupreus	Ccu 4986	UFPA	Left bank of river Madeira, 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 albinasus	CTGAM5663	UFPA	Right bank of river Tapajos

Cacajao calvus	CTGAM5666	UFPA	No information
Pithecia pithecia	Pit 22	UFPA	Left bank of river Jari, Amapá, Brasil

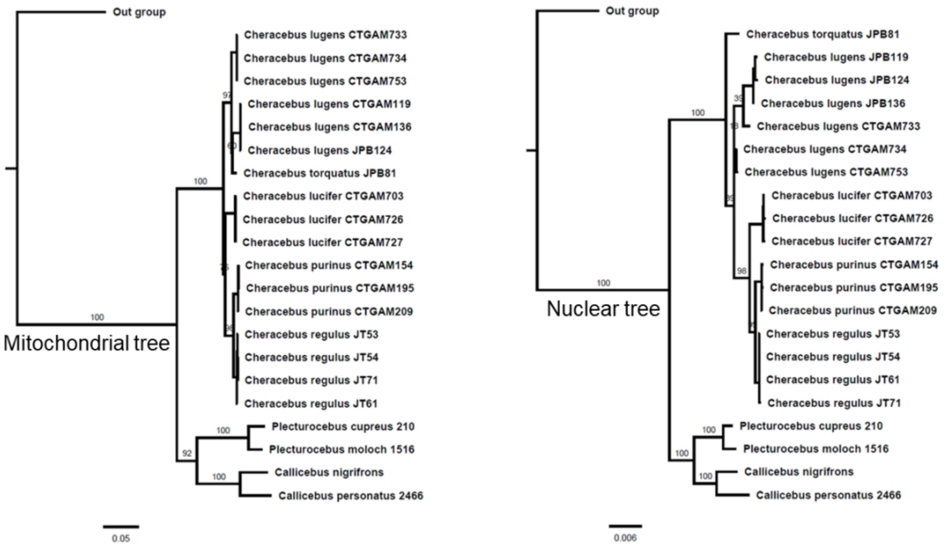
Table 3. Molecular markers used in this study, with their annealing temperatures and references.

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

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* *										
2	*Cheracebus lugens* +	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

* and + mean left and right bank of the river Negro, respectively.



338x190mm (96 x 96 DPI)

Table S1. Markers and their access numbers in GenBank.

Marker	Access number range	
ZFX	MT011236	MT011248
SIM 1	MT011223	MT011235
Alu_Pitheciidae4	MT011205	MT011222
Alu_Pitheciidae3	MT011186	MT011204
Alu_Pitheciidae2	MT011167	MT011185
Pith_Alu1D_84	MT011148	MT011166
Titi_1DF2_39	MT011128	MT011147
Pith_Alu1D_30	MT011113	MT011127
Pith_Alu1D_27	MT011092	MT011112
Cytochrome b	MN998472	MN998495
rRNA 16S	MT002404	MT002424
Cytochrome oxidase I	MN998547	MN998570
Pith_Alu1D_26	MN998449	MN998471
Pith_Alu1D_24	MN998428	MN998448
RAG 1	MN998418	MN998427

Table S2. Data partitioning scheme, markers and respective evolutionary models.

Number of Partitions	Partition names	Evolutionary Models	Numbers of sites
	All molecular markers		
I	Cyt B_pos1, 16S, SIM1, RAG1_pos2, Cyt B_pos2, COI_pos2, Alu84, RAG1_pos3, Alu27, PITH3, RAG1_pos1, DENND5A, COI_pos1, Alu39, PITH2, Alu30, Alu26, ZFX, PITH4, Alu24	TRN+G	9191
II	COI_pos3, CYTB_pos3	TRN+G	564
	Only nuclear markers		
I	Alu24, Alu26, Alu27, Alu30, Alu39, Alu84, DENND5A, PITH2, PITH3, PITH4, RAG1_pos1, RAG1_pos2, RAG1_pos3, SIM1, ZFX	HKY+G	7574
	Only mitochondrials markers		
I	16S, COI_pos1, Cyt B_pos1	GTR+G	1052
II	COI_pos2, Cyt B_pos2	HKY+I	565
III	COI_pos3, Cyt B_pos3	TRN+G	564
	Individual molecular markers		
	16S	GTR+G	486
	COI	GTR+G	623
	Cyt B	GTR+I	1072
	Alu24	GTR	330
	Alu26	GTR	390
	Alu27	GTR+G	636
	Alu30	GTR	693
	Alu39	GTR	431
	Alu84	GTR	480
	DENND5A	GTR	637
	PITH2	GTR	179
	PITH3	GTR	537
	PITH4	GTR	491
	RAG1	GTR+I	1030
	SIM1	GTR	603
	ZFX	GTR	809

Table S3. Genetic distance of nuclear data 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* *										
2	*Cheracebus lugens* +	0.26									
3	*Cheracebus torquatus*	0.37	0.30								
4	*Cheracebus regulus*	1.22	0.40	0.45							
5	*Cheracebus purinus*	0.84	0.65	0.48	0.27						
6	*Cheracebus lucifer*	1.13	0.27	0.47	0.38	0.55					
7	*Plecturocebus*	2.71	3.24	1.47	3.58	2.60	3.56				
8	*Callicebus*	2.95	3.47	1.59	3.76	2.75	3.84	1.64			
9	*Chiropotes*	4.67	6.63	4.05	6.47	4.33	6.47	4.40	4.95		
10	*Cacajao*	4.07	6.92	4.86	6.62	3.89	6.52	3.90	4.16	1.79	
11	*Pithecia*	4.22	6.18	4.48	6.44	4.19	6.31	4.13	4.88	2.25	1.75

* and + mean left and right bank of the river Negro, respectively.

Table S4. Genetic distance of mitochondrial data 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* *										
2	*Cheracebus lugens* +	1.89									
3	*Cheracebus torquatus*	1.91	1.56								
4	*Cheracebus regulus*	3.54	3.32	3.50							
5	*Cheracebus purinus*	3.90	3.63	3.56	1.16						
6	*Cheracebus lucifer*	4.25	3.42	3.36	3.06	3.12					
7	*Plecturocebus*	12.40	12.39	13.10	11.80	12.21	13.41				
8	*Callicebus*	12.22	12.57	13.22	11.89	12.44	13.26	12.13			
9	*Chiropotes*	17.99	18.54	20.14	17.94	18.68	19.83	18.59	18.74		
10	*Cacajao*	17.15	17.26	18.54	16.62	17.40	18.36	18.38	18.03	10.26	
11	*Pithecia*	18.04	18.68	20.13	18.36	18.42	20.14	19.25	18.66	13.27	12.95

Dear Dr. Carneiro,

I thank you for submitting your manuscript AJP-19-0267 entitled "Phylogenetic relationships in the genus <i>Cheracebus</i> (Callicebinae, Pitheciidae)" for review and publication in the American Journal of Primatology. In light of my reading of your paper, as well as the evaluation of your Review Editor and the comments of the external reviewers, I am pleased to inform you that your paper is accepted pending minor revisions.

In addition to addressing the comments below, please include information regarding the ethical approvals for collection of the subject specimens. Specifically, please confirm both that the protocols were approved by the respective institutions, and that the research complied with the American Society of Primatologists Ethical Principles for the Treatment of Non-Human Primates.
R= We incorporated in the manuscript the license number of the collection and that the research followed the ethical principles of American Society of Primatologists.

When submitting your revised manuscript, please provide an itemized response to reviewer(s) comments in the space labeled "Response to Decision Letter." Please note that if you copy and paste your response from a separate document, bold, italicized, and colored text from the original document will appear as black, upright/roman text.

Please make these revisions within two months or less from the date of this letter.

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We thank you for submitting your work to the American Journal of Primatology, and look forward to receiving your revised manuscript.

Sincerely,

Dr. Karen Bales
Editor-in-Chief, American Journal of Primatology
ajpeditorialoffice@wiley.com

EDITOR COMMENTS TO AUTHORS:
Review Editor: Vigilant, Linda
Comments to the Author:

The authors present a focused study on the phylogenetic relationships of the titi monkeys that should be of interest to readers of AJP with a particular interest in primate phylogenies. I find it well-written, but concur with the reviewers that further experimental/analytical detail is needed and also that it is not acceptable to concatenate mitochondrial and nuclear sequences for analyses. Please see the review for detailed suggestions and I look forward to seeing a revised version of the manuscript soon.

REVIEWER COMMENTS TO AUTHORS:

Reviewing: 1

Comments to the Author

The authors investigate phylogenetic relationships among 5 of the 6 species of Cheracebus. The authors can show that Cheracebus is indeed monophyletic and the branching pattern among the species is well resolved. The manuscript is well written, but some rewording is required. Methods and Results are well presented, but I am a little bit concerned about the fact that all analyses are done with a concatenated dataset; thus I recommed to redo some of the analyses.

R= We performed analyzes of mitochondrial and nuclear data separately. Additionally, we carried out a coalescent analysis following the suggestion of the reviewer 2.

Major points:

1. you use a concatenated dataset for all analyses. At least mitochondrial and nuclear data should be analysed separately; this concerns the phylogenetic trees, the dating as well as the genetic distance calculation. Particulary for the distance calculation, one would expect much larger differences in mtDNA compared to nuclear DNA. Trees based on the combined dataset can be presented as main figures and the individual trees in the supplement.

R= We performed analyzes of mitochondrial and nuclear data separately, the trees were included in the supplementary files. We also perform genetic distance analysis with mitochondrial and nuclear data separately.

2. please provide more information about the calibration points for dating: what settings were used in BEAST? Are the 2 points based on fossils, previous molecular dating, etc.? please give here more information. Probably also good to include addtional NWM genera and use more fossil-based calibrations

R= We used a fossil and a calibration based on previous study. We rewrote this part of the text and include the appropriate reference to clarify.

3. I can not find any information about the applied substitution models for the overall dataset or individual loci

R= We made a table that shows all the evolutionary models used in this study.

Minor points:

1. Please check the numbers of your affiliations; they are not in order
2. l32 and l97: based on DNA sequencing of 16
3. l44: 13 million years ago
4. l81: which placed purinus in the
5. l91: Roosmalen et al. (2002)
6. l113: Total genomic DNA
7. l119: 30ng of genomic DNA
8. l122, l125 and Table3: annealing instead of hybridizing/hybridization
9. l127: ethanol instead of alcohol; ... were run with the Big Dye
10. l138: The ML trees
11. l140-2: with two independent Markov chain Monte Carlo (MCMC) runs, with 500,000 generations, and trees and parameters sampled every 5000 generations.
12. l144: estimated with MEGA (xxx) (Tamura et al. 2013).; add also what version was used
13. l147: abbreviation Ma is not explained before
14. l151: LogCombiner v.1.8.3 and TreeAnnotator
15. l163: the clade composed of
16. l191: remove bracket after Callicebus
17. l200: which is consistent eith the morphological data
18. Figures 1 and 2: both can be lumped into one
19. Table1: check arrangements in the Hershkovitz (1990) column
20. Table2: Genebank accession number sare missing; could be added to Table 2 or somewhere else

21. Table3: Mitochondrial instead of Mitochondrials; reference are not in reference list; emty space in reverse primer for RAG1; annealing NOT hybridization

R= Thanks for the corrections, all minor points were corrected.

Reviewing: 2

Comments to the Author

This work is a straightforward analysis of the phylogeny of a new genus of titi monkeys. The authors set out to test the monophyly of the newly proposed Cheracebus genus of neotropical primates. Using a larger sampling of loci, they confirmed earlier taxonomic proposals. Overall, their results are convincing, and the authors avoided going into speculations regarding the biogeography and causes of Cheracebus diversification. Because the subject of the manuscript is rather restricted, it will be of interest mainly to primatologists working on neotropical primate systematics. However, I think this is not a drawback. The only effective shortcoming is the absence of Cheracebus medemi sequences, which prevented the authors to make a de facto evaluation of the monophyly of the genus.

The authors should correct/clarify the following points:

- The authors should justify the concatenation of all loci into a single supergene instead of analyzing them independently. Although it is expected that mitochondrial genes will share the same evolutionary history, nuclear loci may have different histories if they are located either in different chromosomes or distantly enough in the same chromosome (a measure that will depend on the recombination rate). To make their work richer - and to further corroborate their findings - I suggest the authors to run a coalescent-based phylogenetic inference. You can try fast methods such as ASTRAL. There is no need to run a full coalescent inference in BEST, *BEAST or BPP (this will take many days and parameters will likely fail to converge). It might be the case that the coalescent-based phylogeny will be topologically identical to ML/BI. This is fine, because at least the methodological section will be improved: it is reasonable to employ such methods particularly when dealing with shallow divergences.

R= We performed analyzes of mitochondrial and nuclear data separately. Additionally, we carried out a coalescent analysis ASTRAL III.

- Figure 2 should be corrected (text in Portuguese in figure).

R= corriged. A new map was made

- The Methods section needs to be expanded. Please provide detailed information on the model of nucleotide substitution used in ML, BI and BEAST analyses.

R= We made a table that shows all the evolutionary models used in this study.

- Which node was calibrated by "the pitheciine fossil, Nuciruptor rubricae (Meldrum & Kay, 1997), dated to 14812.4–12.8 Ma." Was it the root node?

R= We used a fossil and a calibration based on previous study. We rewrote this part of the text and include the appropriate reference to clarify.

- "The 16 nuclear and mitochondrial markers provided a database of 9755 base pairs (bps), 2300 bps from the mitochondrial sequences, and 7455 bps from the nuclear sequence". An alignment of 9755 base pairs (bps)?

R= Yes, it is the alignment of the concatenated loci. We corrected that part of the text, the complete alignment actually has 9427 base pairs.

- "All the species were identified as monophyletic". I suggest using "All allelic diversity within species was reciprocally monophyletic".

R= We made the suggested change

- "is virtually the same as that of the first diversification within the callicebines" --> close to the first?

R= there was a mistake. We wanted to refer to another node. The Split of *Pithecia* from the other pithecineos (*Cacajao* and *Chiropotes*).

- "are the species with the shortest divergence time" --> earliest divergence time?

R= We were referring to the most recent speculations within the genus Cheracebus. We rewrote to clarify

- Please clarify what you mean by "patterns of genetic drift".

R= We referred to random genetic drift events in different species. But we decided to remove that part of the text