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**Phylogeny, molecular dating and zoogeographic history of the titi monkeys (*Callicebus*,  
*Pitheciidae*) of eastern Brazil**

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## **Abstract**

The titi monkeys belong to a genus of New World primates endemic to South America, which were recently reclassified in three genera (*Cheracebus*, *Plecturocebus* and *Callicebus*). The genus *Callicebus*, which currently includes five species, is endemic to eastern Brazil, occurring in the Caatinga, Savanna, and Atlantic Forest biomes. In the present study, we investigated the validity of these species and inferred their phylogenetic relationships, divergence times, and biogeographic patterns based on the molecular analysis of a concatenated sequence of 11 mitochondrial and nuclear DNA markers, derived from 13 specimens. We ran Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, and estimated genetic distances, divergence times. Ancestral areas were estimated on BioGeoBears. Our results suggest that at about twelve million years ago, the ancestor of all titi monkeys inhabited a wide area that extended from the Amazon forest to the South of the Atlantic forest. A first vicariant event originated *Cheracebus* in the West of the Amazon and the ancestor of *Callicebus* and *Plecturocebus* which, later were separated by a second one. The diversification of *Callicebus* occurred during the Plio-Pleistocene (beginning at 5 Ma) probably influenced by climatic fluctuations and geological events. Therefore, the results of the present work confirmed the existence of five species that currently inhabit forested areas under increasing threat from human activities. Thus, a reliable diagnosis of the taxonomic status of species living in endangered environments is extremely important for the development of conservation measures.

**Key words:** *Callicebus*, New World monkeys, taxonomy, dispersal, zoogeography, phylogeny



## 1. Introduction

The titi monkeys, subfamily Callicebinae Thomas 1903, are New World primates of the family Pitheciidae (Groves, 2001). In volume XII of the *Annals and Magazine of Natural History*, Thomas (1903) proposed the genus *Callicebus* for the titi monkeys, which were previously assigned to the genera *Cebus*, *Saimiri*, and *Callithrix* (Elliot, 1913). In the subsequent decades, Elliot (1913), Cabrera (1957) (1958), and Hill (1960) listed 22–34 species. Following an extensive review of the morphological traits of specimens obtained in the Amazon and Orinoco basins, however, Hershkovitz (1963) restricted the genus to two species, containing a total of 10 subspecies, although this same author (1988, 1990) subsequently revised his classification, based on a more comprehensive set of data, recognizing 13 species and 25 taxa, which were assigned to four species groups (*donacophilus*, *torquatus*, *moloch* and *modestus*). Kobayashi (1995) proposed a number of modifications to this arrangement, based on an extensive analysis of morphometric, cytogenetic, and zoogeographic data, dividing the *moloch* group into three (*moloch*, *cupreus* and *personatus*), and reallocating *modestus* to the *donacophilus* species group. Based on molecular data, Carneiro et al. (2016) questioned the validity of the *cupreus* group, recommending its inclusion in the *moloch* group.

Recently, Byrne et al. (2016) analyzed approximately 14 Kb of DNA sequences, and proposed a new arrangement for the titi monkeys, which they allocated to three genera – *Cheracebus* (*torquatus* group), *Plecturocebus* (*moloch/cupreus* and *donacophilus* groups) and *Callicebus* (*personatus* group). We have adopted this new classification in the present study.

*Cheracebus* and *Plecturocebus* occur primarily in the Amazon basin and some adjacent areas (Fig. 1). The five species of the genus *Callicebus*, *Callicebus personatus* Geoffroy 1812, *Callicebus melanochir* Wied-Neuwied 1820, *Callicebus nigrifrons* Spix

1823, *Callicebus barbarabrownae* Hershkovitz 1990 and *Callicebus coimbrai* Kobayashi and Langguth 1999, are endemic to eastern Brazil, centered on the Atlantic Forest, and adjoining areas of the Caatinga and Savanna biomes.

As for most New World primates, the *Callicebus* species are recognized primarily on the basis of the coloration of the pelage (Hershkovitz, 1990; van Roosmalen et al., 2002), even though considerable intraspecific variation may be found in this trait, which hampers reliable identification, and in particular the definition of the limits of species ranges (Cardoso de Sousa et al., 2008; Kobayashi, 1995; Kobayashi and Langguth, 1999; Printes, 2011). Based on a geometric analysis of cranial morphology, Kobayashi (1995) and Kobayashi and Langguth (1999) assigned the *Callicebus* species to a single clade, together with the Amazonian *Plecturocebus dubius* and *Plecturocebus brunneus*, a configuration not supported by the analysis of Byrne et al. (2016). None of the previous arrangements proposed for this group included analyses of specimens of all five of the currently-recognized species, considering that *Callicebus coimbrai* was only described at the turn of the 20th Century (Kobayashi and Langguth, 1999).

Given these differences in the interpretation of the diversity of the genus, the present study investigated the validity of the classification of the five *Callicebus* species, and provides insights into the phylogenetic relationships among the different forms, including the chronology of their diversification and possible patterns of dispersal. The findings of this study are important not only for the understanding of the evolutionary history of the titi monkeys, but also for the conservation of the genus, given that *C. barbarabrownae* is listed as critically endangered, *C. coimbrai* as endangered, while *C. personatus* and *C. melanochir* are considered to be vulnerable to extinction (IUCN, 2016).

## 2. Material and Methods

We analyzed 13 samples (one of each of the following species: *C. coimbrai*, *C. barbarabrownae*, *C. melanochir*, *C. nigrifrons*, *C. personatus*, *Plecturocebus moloch*, *Plecturocebus cupreus*, *Plecturocebus brunneus*, *Cheracebus lugens*, *Cheracebus purinus*, *Chiropotes albinasus*, *Cacajao calvus*, *Pithecia pithecia*) from blood or muscular tissue preserved in absolute alcohol, obtained from the Federal University of Para (UFPA), Federal University of Amazonas (UFAM), the National Institute for Amazonian Research (INPA), the Rio de Janeiro Primatology Center, and the Getulio Vargas Zoobotanical Park in Salvador. The specimens were identified based on their external morphology, i.e., pelage coloration pattern, following van Roosmalen et al. (2002), in addition to the available data on the geographical locality from which the specimen was obtained (Table 1).

Total DNA was extracted using the Wizard Genomics kit (Promega Corporation, Madison, WI, USA), according to the maker's recommendations. The markers were amplified by Polymerase Chain Reaction (PCR) using the following the protocol: initial denaturation at 95°C for four minutes, followed by 35 cycles of (i) 30 seconds for denaturation at 95°C, (ii) 45 seconds for the hybridization of the primers at the specific temperature (Table S1), and (iii) 30 seconds of extension at 72°C. At the end of these 35 cycles, there was a final extension of 5 minutes at 72°C. The final volume of the PCRs was 15 µl, containing approximately 50 ng of genomic DNA, 2.4 µl of dNTPs (1.25 mM), 1.5 µl of 10x Buffer (200 mM Tris-HCl, 500 mM KCl), 1 µl of MgCl<sub>2</sub> (25 mM), 1 µl of each primer (0.2 µM), and 1 U of Taq DNA polymerase. The PCR products were purified using polyethylene glycol and ethanol (Paithankar and Prasad, 1991). The sequencing reactions were run using the Big Dye sequencing kit V. 3.1 (Life Technologies, Carlsbad, CA, USA),

and the products were transferred into ABI 3500xl automatic sequencer (Life Technologies, Carlsbad, CA, USA).

We used 11 molecular markers (see Table S1), three mitochondrial genes (16S, cytochrome oxidase I – COI, and cytochrome *b* - Cyt *b*), four *Alu* elements together with their flanking regions, and four autosomal genes (ATP-binding cassette transporter A1-ABCA1, DENN domain containing 5A - DENND5A, c-FES proto oncogene - FES and Recombination activating gene 1 - RAG1). The sequences were aligned in ClustalW (Thompson et al., 1994) and corrected manually in BioEdit v. 7.2.5 (Hall, 1999). An independent evolutionary model was estimated for each molecular marker in the Kakusan software, v. 4-4.0.2015.01.23 (Tanabe, 2011) see Table S1). We estimated the genetic distances between taxa in the Molecular Evolutionary Genetics Analysis software, MEGA v. 6.0 (Tamura et al., 2013), using the K2P model.

We used Maximum Likelihood (ML) and Bayesian Inference (BI) approaches for the phylogenetic reconstruction. The ML analysis was run in RAxML v. 8 (Stamatakis, 2014) with 1000 bootstrap replications, while the BI was run in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003) with two independent runs using four Markov Monte Carlo chains (MCMC), one cold and three hot, with 400,000 generations, with trees and parameters being sampled every 500 generations, and 25% of the runs being discarded as burn-in. We evaluated the convergence of the runs visually in Tracer v. 1.6 (Rambaut et al., 2014). All the values of Effective Sample Size (ESS) were over 200.

The estimates of divergence time and the species tree were produced simultaneously in BEAST 1.8.3 (Drummond et al., 2012). To calibrate the estimates of divergence times, we used a soft maximum bound of 13.6 Ma, derived from the fossil genus *Miocallicebus* (Takai et al., 2001) and applied a calibration of 6.7 Ma (SD = 2.3), which represents the estimate of the divergence time between *Cacajao* and *Chiropotes* (Byrne et al., 2016). Uncorrelated

relaxed clocks were attributed to estimates of branch lengths, and the prior Yule was used for the tree as a whole. We used four independent runs of  $20^7$  generations, with parameters and trees being sampled every 2500 generations. The convergence of the runs was evaluated in Tracer v. 1.6 (Rambaut et al., 2014) for ESS values of above 200. The results of the trees and the log files were summarized, respectively, in TreeAnnotator v. 1.8.3 and LogCombiner v. 1.8.3, both run in Beast 1.8.3 (Drummond et al., 2012), with a 20% burn-in being used in each case. The tree was visualized and edited in FigTree v. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Biogeographic analyses were performed in BeoGeoBEARS (Matzke, 2013). Six models (DEC, DECj, DIVA, DIVAj, BayArea, BayAreaj) were tested hoping reconstructing the ancestral areas of the titi monkeys. The best model was chosen by the Akaike Information Criterion. First, four biogeographic areas were supplied: Western Amazonia (WA); Eastern Amazônia (EA) limited by Madeira River; North Atlantic forest (NA) and South Atlantic forest (SA) limited by Doce River (Fig. 2). Therefore, the number limit of four areas for each node in the cladogram was implemented.

## Results

We obtained a data base of 6965 base pairs (bps), including 4772 nDNA and 2193 mtDNA. The topologies of the ML, IB and species trees were the same (Fig. 3). The BI tree presented maximum values of *a posteriori* probability for all the nodes, whereas in the species tree, only three nodes returned the maximum probability, and in the case of the ML analysis, all but two nodes returned maximum values (Table 2). The titi monkeys are a clear monophyletic group, and sister group of the Pitheciinae (*Pithecia*, *Chiropotes* and *Cacajao*). Three principal clades (*Cheracebus*, *Plecturocebus* and *Callicebus*), all monophyletic, were recuperated from the titi monkey lineage (see Fig. 3). The first of these three clades split off

at 12.5 Ma (95% Highest Posterior Density [HPD]: 11.4–13.6 Ma), originating *Cheracebus* and the ancestral *Plecturocebus/Callicebus*, which diverged at 9.86 Ma (95% HPD: 8.4–10.9 Ma: Fig. 3). The mean genetic distance between *Cheracebus*, *Plecturocebus* and *Callicebus* is approximately 12% for the concatenated markers (Table S2). The genetic distances for the mitochondrial and nuclear markers, analyzed separately, are shown in Table S3.

Within the genus *Callicebus*, our estimates indicate that *C. nigrifrons* was the first lineage to diverge, around 5.5 Ma (95% HPD: 4.4–6.5 Ma), followed by *C. personatus*, at 3.2 Ma (95% HPD: 2.5–4.1 Ma), and then *C. melanochir*, at 1.9 Ma (95% HPD: 1.4–2.5 Ma). The last lineages to diverge were *C. barbarabrownae* and *C. coimbrai*, at around 1.1 (95% HPD: 0.7–1.6 Ma). The interspecific distances range from 1.3% to 6.5%, with a mean of 4.6% for the concatenated sequence (Table S3).

Of the six models available on BioGeoBears (DEC, DECj, DIVA, DIVAj, BayArea, and BayAreaj) DIVAj was the model that produced the highest AIC score and therefore was the best model supported by the data (Table S4). This model suggests that the most recent common ancestor of *Cheracebus*, *Plecturocebus* and *Callicebus* occupied a wide geographical area in South America, occurring from the Amazon to the Atlantic Forest.

In addition, our results indicate that the first diversification event of titi monkeys occurred in the Western Amazonia at about 12Ma originating *Cheracebus* and the ancestral of *Callicebus* and *Plecturocebus*. Later a cladogenesis event occurred restricting *Callicebus* to the Atlantic Forest, but specifically in the region south of the Doce River and *Plecturocebus* in the Eastern Amazonia and Chaco (Fig. 2).

The present *Callicebus* species began to diversify at the beginning of the Pliocene giving rise to the *C. nigrifrons* and *C. personatus* species. Later, a lineage of *Callicebus* occupied a region to the north of the Doce River and by founder effect originated the ancestor of the present *C. melanochir*, *C. barbarabrownae* and *C. coimbrai*.

### 3. Discussion

While the titi monkeys were classified in five species groups (*torquatus*, *donacophilus*, *moloch*, *cupreus* and *personatus*) over the past 20 years (Kobayashi, 1995), Byrne et al. (2016) revolutionized the systematics of this group using molecular data, and proposing three genera, *Cheracebus* (= *torquatus* group), *Plecturocebus* (= *donacophilus* + *moloch* + *cupreus* groups), and *Callicebus* (= *personatus* group). While the present study was based on different molecular markers and calibration parameters from those used by Byrne et al. (2016), our findings on the phylogenetic relationships and divergence times among the three titi genera (*Cheracebus*, *Plecturocebus* and *Callicebus*) are broadly consistent with the results of this earlier study. The matrices of genetic distances between the three genera, based on multilocus data (see Tables S2 and S3), also provide important insights into the associations among these three closely-related genera.

The taxonomy of the genus *Callicebus* was based traditionally on the variation in the coloration of the pelage (Hershkovitz, 1988, 1990; van Roosmalen et al., 2002), and while Hershkovitz (1988, 1990) acknowledged the existence of the different forms (except *C. coimbrai*, which was described subsequently by Kobayashi and Langguth (1999), he classified them as subspecies of a single species, *C. personatus*. Our estimates of the genetic distances between these forms (Table S3) contradict this proposal, and support the findings of Kobayashi and Langguth (1999), based on the analysis of cranial morphometrics, although it is important to note the relatively reduced distance (1.3%) recorded between *C. coimbrai* and *C. barbarabrownae*, which reflects the relatively recent separation ( $1.1 \pm 0.7$ –1.6 Ma) of these lineages. However, these two forms are ecologically distinct, with *C. barbarabrownae* being restricted to the Caatinga dry forests and *C. coimbrai* to the more humid coastal Atlantic

Forest, with recent surveys (Ferrari *et al.*, 2013) showing that they may occupy completely allopatric ranges in these two biomes.

Herskovitz (1990) noted clinal variation in the coloration of the head of the different *Callicebus* forms (which he classified as subspecies), ranging from black agouti to whitish, although the first systematic insights into the phylogenetic relationships of these titis were provided by the morphometric analyses of Kobayashi (1995) and Kobayashi and Langguth (1999), followed more recently by the molecular study of Byrne *et al.* (2016). The results of the phylogenetic analysis obtained in the present study contradict the patterns observed in the morphological studies of both Herskovitz (1990) and Kobayashi and Langguth (1999), but are at least partly consistent with the more recent molecular findings.

Paleoclimatic analyses (Micheels *et al.*, 2007; Sobral-Souza *et al.*, 2015) indicate that the Amazon and Atlantic forests were connected during the Paleogene, but were separated by the Savannas, extending from northeastern Brazil to Paraguay, between the middle and late Miocene.

This scenario is in agreement with our biogeographic findings, which suggested that the ancestors of titi monkeys occupied a wide geographic area in South America, taking advantage of the connectivity between the Amazon and Atlantic Forest. Nevertheless, after the emergence of extensive areas of Savannas between Amazon and Atlantic forests, *Callicebus* became restricted to the Atlantic Forest, and therefore, isolated from Amazonian titi monkeys.

Paleoclimatic analyzes (Micheels *et al.*, 2007, Sobral-Souza *et al.*, 2015) indicate that the Amazon and Atlantic forests were connected during the Paleogene, but between the middle and late Miocene they were separated by a huge area of savanna, extending from northeastern Brazil to Paraguay. This scenario is consistent with our biogeographic results, because the ancestral populations of the titi monkeys could have inhabited a wide geographic

area in South America, taking advantage of the connectivity between the Amazon and Atlantic Forest. However, after the rise of the extensive savanna areas between Amazon and Atlantic forests the *Callicebus* populations were restricted to the Atlantic Forest and, therefore, isolated from Amazonian titi.

Recent molecular studies (Alfaro et al., 2015; Kiesling et al., 2015) have pointed to an Amazonian origin for most of the current genera of New World monkeys. Our biogeographic estimate suggests for the crown lineage of the titi monkeys one origin at about 12 Ma, before the separation between Amazon and Atlantic forests (Fig. 3). Later, a second radiation originated *Callicebus* in the Atlantic forest and *Plecturocebus* in the Amazon forest, such as today the distribution of *Callicebus* is now centered on the Atlantic Forest of southeastern Brazil. This event coincides with the origin and development of the Savanna biome about 10 Ma.

Besides that, our estimates suggest that the most recent common ancestor (MRCA) of *Callicebus* diversified around 5.5 Ma, agreeing with the transition of Miocene to Pliocene.

The biogeographic estimates also indicate that the first divergence of the genus *Callicebus* originated *C. nigrifrons* and the ancestor of the remaining titis in the southern Atlantic Forest. This event was followed by *C. personatus* speciation still at the South of Atlantic forest. On the other hand, the origin of the three species located to the North of the Atlantic forest (*C. coimbrai*, *C. barbarabrownae*, and *C. melanochir*) is probably related to an ancient overcoming of the barrier represented by the Doce River which may have occurred in the beginning of the Pleistocene.

The hypothesis of a past connection between Amazonia and the southern Atlantic Forest is also supported by the present-day distribution of plant species typical of both forests in the region's Savanna (Méo et al., 2003). In fact, the phytophysiognomy of the Savanna is

extremely complex, including grassland and woodland formations (Batalha, 2011), and while the gallery forests of this biome are now mostly discontinuous, it seems likely that past climate fluctuations, such as those occurring during the Late Miocene, may have influenced their connectivity significantly.

A number of centers of endemism have been identified in the Atlantic Forest, based on the similarities of the distribution patterns of diverse groups of mammals, birds, and invertebrates (Cabanne et al., 2008; Carnaval et al., 2014; Thomé et al., 2010). The Atlantic Forest is a biodiversity hotspot, and geological and/or climatic processes are frequently used to account for this biological richness. Both types of process may have influenced the radiation of *Callicebus* in the Atlantic Forest. For example, the Doce River in Espírito Santo and Minas Gerais is the current limit of the ranges of *C. nigrifrons* and *C. personatus*, and may have played an important role in the divergence of *C. nigrifrons* from the ancestor of the other *Callicebus* species. A large number of studies have identified the Doce River as one of the most important barriers to gene flow in the Atlantic Forest, through the separation of closely-related sister taxa (Carnaval et al., 2014; da Silva and Casteleti, 2003; França et al., 2015; Resende et al., 2010; Valdez and D'Elía, 2013).

On the other hand, *C. coimbrai* and *C. barbarabrownae* are not separated by any physical barrier to dispersal, although the semi-arid climate of the Caatinga, and the limited availability of suitable habitats, such as cloud forest refuges (Printes, 2011), may have contributed to the ecological isolation of the two species over a relatively recent time scale (Ferrari *et al.*, 2013). The more humid habitats found within the Caatinga may represent remnants of the periods during the Pleistocene when the climate was cooler (by as much as 5°C) and wetter (De Oliveira et al., 1999). The current differences in the morphology and genetics of *C. coimbrai* and *C. barbarabrownae* may thus have been molded by their relatively recent ecological diversification.

Unfortunately, the distribution of these species also coincides with some of the most impacted habitats in eastern Brazil, which have suffered the onslaught of European colonization ever since the first landfall, at the very beginning of the 16th Century (Saatchi et al., 2001). With the loss of habitat exceeding 90%, the remaining titi populations are restricted to small, isolated fragments of forest (Jerusalinsky et al., 2006; Marques et al., 2013; Printes, 2011), where they are extremely vulnerable to extinction. While conservation efforts are urgently needed throughout the distribution of the genus, the reliable diagnosis of species-level, as provided by the present study, is a fundamental component of any management program.

#### **4. Conclusion**

We used DNA sequences from different portions of the genome (mtDNA, *Alus* and genes) obtained from specimens of all five *Callicebus* species to confirm the monophyly of the genus and infer the phylogenetic relationships among species. The results of the study confirmed the validity of all currently-recognized species. We also provide the first detailed biogeographical analysis of the genus, with estimates of divergence time, which indicate that the *Callicebus* lineage split from the Amazonian titi monkeys during the late Miocene, when the Savanna arise. During the Plio-Pleistocene other species diverged possibly in the context of climate fluctuations and geological events.

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## Figure Captions

**Fig. 1.** Geographical distribution of titi monkeys. Modified from ([Byrne et al., 2016](#)).

Area marked with vertical bars indicates sympatry area between *Cheracebus* and *Plecturocebus*.

**Fig. 2.** Consensus divergence time and estimation of ancestral areas, and map showing the geographic regions chosen as possible ancestral areas in the BioGeoBears analysis.

**Fig. 3.** Topologies of maximum likelihood (ML), Bayesian inference (IB) and species-tree (ST) trees. Numbers above the nodes are related to the values of bootstrap support (ML) and posterior probability (PP) for BI. Below the cladogram is shown a timescale with emphasis on three geological epochs related to the present study.

**Table S1.** Molecular markers used in the present study. Details of annealing temperature of PCR, evolutionary models are also shown.

Molecular Markers	Code	Primers		Annealing Temp. °C	Evolutionary models	References
		Forward	Reverse			
16S rRNA	16S	5'TGGACTATGAGTTGAGCAGAC3'	5' TATGCTAATTACTCTTCTTGGGC 3'	58	GTR+GAMMA	Palumbi 1991
Cytochrome oxidase I	COI	5' TCCATTACCAGGCCAGCTAG3'	5' GAACTTGCTGGCTTTCATATC 3'	45	HKY+GAMMA	Ward et al. 2005
Cytochrome <i>b</i>	<i>Cyt b</i>	5' GCACCTACCCACGAAAAGAA3'	5' ACATTGCCTCTGCAAATTGA 3'	62	HKY+GAMMA	Carneiro et al., 2016
Alu Pitheciidae	Pith_AlulD_24	5' AAGCCATAACTCCATTACCAAA3'	5' AGATTCTGGTCCCAAGTCCA 3'	60	HKY	Ray et al. 2005
Alu Pitheciidae	Pith_AlulD_27	5' AACACATTTTACTGTATGCTG3'	5' CCCTTCAATGACTCCCTTCA 3'	57	GTR+GAMMA	Ray et al. 2005
Alu Pitheciidae	Pith_AlulD_84	5' CTGCTACGTCAGACGTCGTAC3'	5' CTGCTAGCACAAAGCTAGTCGA 3'	62	HKY+GAMMA	Ray et al. 2005
Alu titi monkeys	Titi_1DF2_39	5'AACAGAGTTGGCCGTTTCATCT3'	5' GTCCTGTTCAAGTCAGCTACGTTG 3'	54	K80+GAMMA	Ray et al. 2005
ATP-binding cassette transporter A1	ABCA1	5'CCTCCATCTTTTCAGCTCTACCTAC3'	5' ACAAGAGCCTGGAGATTGGATAAC 3'	48	HKY+GAMMA	Hovath et al., 2008
DENN domain containing 5A	DENND5A1	5'CCAGAGTTATCATGGCCAATC3'	5' GAAAACCTGGCCACAGTTGA 3'	48	K80+GAMMA	Perelman et al., 2011
c-FES proto oncogene	FES	5'GGGGAACTTTGGCGAAGTGTT3'	5' TCCATGACGATGTAGATGGG 3'	47	HKY+GAMMA	Venta et al., 1996
Recombination activating gene 1	RAG1	5'GCTTTGATGGACATGGAAGAAGACAT3'	5' GAGCCATCCCTCTCAATAATTTTCAGG 3'	47	K80+GAMMA	Teeling et al., 2000

**Table S2.** Genetic divergence (%) between the genera of Pitheciidae family estimated based on data from Table S3.

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
<b>1</b> <i>Cheracebus</i>					
<b>2</b> <i>Plecturocebus</i>	12.6				
<b>3</b> <i>Callicebus</i>	12.2	12.3			
<b>4</b> <i>Chiropotes</i>	18.3	18.8	18.4		
<b>5</b> <i>Cacajao</i>	17.4	18.8	18.1	4.1	
<b>6</b> <i>Pithecia</i>	18.6	19.6	19.2	13.6	13.2

**Table S3.** Genetic divergence (%) between specimens analyzed in the present study.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>1</b> <i>Callicebus nigrifrons</i>												
<b>2</b> <i>Callicebus personatus</i>	6.5											
<b>3</b> <i>Callicebus melanochir</i>	6.3	5.1										
<b>4</b> <i>Callicebus coimbrai</i>	6.1	4.9	2.4									
<b>5</b> <i>Callicebus barbarabrownae</i>	6.1	5.1	2.3	1.3								
<b>6</b> <i>Plecturocebus brunneus</i>	12.4	12.7	12.5	12.5	12.8							
<b>7</b> <i>Plecturocebus cupreus</i>	11.6	11.6	11.8	12.2	12.3	3.3						
<b>8</b> <i>Plecturocebus moloch</i>	12.0	12.7	12.3	12.5	12.5	3.6	3.9					
<b>9</b> <i>Cheracebus purinus</i>	12.4	13.0	12.3	11.7	12.0	13.0	12.3	12.3				
<b>10</b> <i>Cheracebus lugens</i>	12.5	12.7	12.3	11.3	11.9	13.3	12.6	12.0	3.9			
<b>11</b> <i>Chiropotes albinasus</i>	18.5	19.2	18.5	17.6	18.3	19.1	18.6	18.7	18.7	17.9		
<b>12</b> <i>Cacajao calvus</i>	18.0	18.5	18.2	17.5	18.1	19.1	18.7	18.5	17.5	17.2	4.1	
<b>13</b> <i>Pithecia pithecia</i>	19.1	18.9	19.2	19.1	19.6	19.7	19.9	19.4	18.7	18.5	13.6	13.2

Table S4. BioGeoBEARS model comparison based on log-likelihood (lnL); n, number of parameters; d, rate of dispersal; e, rate of extinction; j, relative probability of founder-event speciation, and the Akaike information criterion (AIC) and AIC weight.

	LnL	n. params	d	e	j	AIC	AIC weight
DIVALIKE+J	-9.126	3	$10^{-12}$	$10^{-12}$	0.09	24.25	0.77
DEC+J	-9.495	3	$10^{-12}$	$10^{-12}$	0.08	24.99	0.90
BAYAREALIKE+J	-10.478	3	$10^{-07}$	$10^{-07}$	0.10	26.96	1.00
DIVALIKE	-11.353	2	$1.4^{-02}$	$10^{-12}$	0.00	26.71	0.23
DEC	-12.687	2	$7.6^{-03}$	$10^{-12}$	0.00	29.37	0.10
BAYAREALIKE	-17.815	2	$1.5^{-02}$	$1.2^{-01}$	0.00	39.63	0.00

**Table 1.** Details of the specimens analyzed in the present study, including their, code, origin and collecting locality.

<b>Taxa</b>	<b>Code</b>	<b>Origin</b>	<b>Locality</b>
<i>Callicebus coimbrai</i>	Cc01	PZGV	NI
<i>C. barbarabrownae</i>	Cb01	PZGV	NI
<i>C. melanochir</i>	CPRJ2329	CPRJ	Eunápolis, Bahia, Brazil
<i>C. nigrifrons</i>	Cpe04	PUC	Minas Gerais, Brazil
<i>C. personatus</i>	CPRJ2466	CPRJ	Aracruz, Espirito Santo, Brazil
<i>Plecturocebus moloch</i>	MCB79	UFPA	Senador José Porfírio, right bank of the Xingu River, Pará, Brazil
<i>P. cupreus</i>	Cbr4981	UFPA	UHE Samuel, right bank of the Jamari River, Rondônia, Brazil
<i>P. brunneus</i>	Cbr2220	UFPA	UHE Samuel, right bank of the Jamari River, Rondônia, Brazil
<i>Cheracebus lugens</i>	JPB 119	INPA	Marari, Amazonas, Brazil
<i>C. purinus</i>	CTGAM195	UFAM	Rebio Abufari, left bank of the Rio Purus, Amazonas, Brazil
<i>Chiropotes albinasus</i>	CTGAM5663	UFPA	NI
<i>Cacajao calvus</i>	CTGAM5665	UFPA	NI
<i>Pithecia pithecia</i>	Pit22	UFPA	NI

PZGV - Parque Zoobotanico Getúlio Vargas; CPRJ - Rio de Janeiro Primate Center; PUC - Pontifical Catholic University; UFPA - Federal University of Para; UFAM - Federal University of Amazonas; INPA - National Institute for Amazonian Research; NI - No Information; UHE - Hydroelectric Plant.

**Table 2.** Nodes Supports, nodes ancestral range and divergence times corresponding to Fig. 3.

<b>Node</b>	<b>BS ML</b>	<b>PP IB</b>	<b>PP SpeciesTree</b>	<b>Mean (Ma) DT</b>	<b>SD Lower 95% HPD</b>	<b>SD Upper 95% HPD</b>
1	100	1	1	20.4	18.7	22.1
2	100	1	1	12.9	10.6	15.42
3	100	1	1	12.5	11.4	13.6
4	100	1	1	5.6	5.2	6.8
5	100	1	1	3.1	2.2	4.2
6	100	1	1	9.8	8.4	10.9
7	100	1	1	3.4	2.6	4.2
8	100	1	1	5.5	4.4	6.5
9	81	1	0.94	2.1	1.4	2.5
10	100	1	1	3.2	2.5	4.1
11	100	1	0.98	1.9	1.4	2.5
12	96	1	0.93	1.1	0.7	1.6

BS: Bootstrap; PP: Posterior probability; SD: Standard Deviation; DT: Divergence Time





