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# Mitochondrial Divergence Between 2 Populations of the Hooded Capuchin, *Cebus (Sapajus) cay* (Platyrrhini, Primates)

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## Abstract

We analyzed the molecular divergence of 2 separate populations of *Cebus apella paraguayanus*, recently considered a junior synonym of *Cebus cay*, and estimated its time of separation from *C. apella*. Cytochrome *b* DNA from 23 *C. cay* from Brazil and 9 from Paraguay showed 24 haplotypes (20 and 4, respectively), accounting for 29 variable sites (19 transitions and 10 transversions), with 40.0%, 26.7%, and 33.0% replacements at first, second, and third codon positions, respectively. Genetic distance between haplotypes averaged 0.5%, with 1.1% between *C. cay* populations. Phylogenetic reconstructions and median joining separated *C. cay* from Brazil and Paraguay. Neighbor joining showed *C. cay* and *C. apella* as sister groups, although *C. cay* and *C. apella* collapsed in maximum parsimony and maximum likelihood topologies. Analysis of molecular variance showed the highest variance component between *C. cay* populations, and mismatch distribution indicated that this species suffered a recent demographic expansion. Divergence time estimates suggested that the 2 populations of *C. cay* split in the Pleistocene, a period of repeated glaciation events leading to drastic changes in the vegetation composition of different biomes.

**Key words:** Cebus cay, *cytochrome* b, *mitochondrial DNA*

*Cebus* (Erxleben 1777) is a widespread genus with a long evolutionary history in the New World (Hill 1960) and accounting for one of the most controversial taxa of Neotropical mammals (Cabrera 1917; Silva Jr. 2002). Hershkovitz (1949) separated *Cebus* into 2 groups based on the presence of frontal hair clusters. These included the tufted group, represented by a single species, *Cebua apella* (Linnaeus 1758) and the un-tufted group comprising *Cebus capucinus* (Linnaeus 1758), *Cebus albifrons* (Humboldt 1812) and *Cebus nigrivittatus* Wagner 1848.

A recent revision (Silva Jr. 2001; 2002) recognized the tufted and untufted groups as subgenera based on their morphologic characteristics and geographic distribution. According to this taxonomic arrangement, the subgenus *Cebus* Erxleben, 1777 comprises four species of the un-tufted group: *Cebus (cebus) capucinus* (Linnaeus 1758), *Cebus (cebus) albifrons* (Humboldt 1812), *Cebus (Cebus) olivaceus* Schomburgk, 1848, and *Cebus (Cebus) kaapori* Queiroz, 1992. The subgenus *Sapajus* Kerr, 1792 comprises the following tufted species: *Cebus (Sapajus) apella* (Linnaeus 1758), *cebus (Sapajus) macrocephalus* Spix, 1823, *Cebus* (*Sapajus*) *libidinosus* Spix, 1823, *Cebus (Sapajus*) *cay* Illiger, 1815, *Cebus (Sapajus) xanthosternos* Wied, 1820, *Cebus (Sapajus) robustus* Kuhl, 1820, *Cebus (Sapajus) nigritus* (Goldfuss 1809), and the neotype *Cebus (Sapajus) flavius* (Schreber 1774) designated for *simia flavia* (Oliveria and Langguth 2006).

Illiger (1815) described *Cebus apella cay* based on the original description of Azara (1809). Its type locality,

originally in Paraguay, was delimited by the east bank of the Rio Paraguay (Hill 1960). According to Cabrera (1917), *Cebus azarae* (Rengger 1830) was the same as *Cebus paraguayanus* (Fischer 1829), a species found at the type locality previously assigned to *C. apella cay* by Hill (1960), and recognized by Silva Jr. (2001) as a junior synonym of *C.* (*Sapajus*) *cay*. This species is distributed in west central Brazil, delimited by transitional regions between the Amazon Rainforest and the Cerrado domains to the north and northwest of its range, by the Rio Araguaia to the east, and by the Rio Paraná to the south, extending to the northwest of Paraguay and Argentina (Silva Jr. 2001). The distribution of *C. cay* is widespread throughout different domains like Cerrado, Pantanal, part of Chaco, and the Atlantic and Amazonian Forests, as well as the Andean Yungas (Silva Jr. 2001; see Figure 1).

There is no available data, however, on the time this species split from its common ancestor with *C. apella* or on the divergence of *C. cay* across its extensive geographic distribution, comprising separate populations under the selective pressures of different morphoclimatic domains. Several karyologic and biochemical studies of *C. cay* (*Cebus apella paraguayanus*) have been carried out (Matayoshi et al. 1986; Schneider et al. 1988; Sampaio et al. 1991; Mudry et al. 2007), although population studies of *C. cay* have not been reported to present. Our study intends to elucidate when this species emerged and how 2 separate populations diverged from one another.

Phylogeographic studies of other neotropical primates have shown that rivers might represent isolating geographic barriers, as was the case of the *Saguinus niger* populations across the Rio Tocantins (Pará state, Brazil). Analysis of the D-loop region of the mitochondrial genome showed a higher divergence between populations of different banks than between valid species like *Saguinus mystax* and *Saguinus imperator* (Vallinoto et al. 2006). Similarly, phylogenetic reconstructions of *Callicebus lugens* from the north and south banks of the Rio Negro (Amazonas state, Brazil), based on cytochrome *b* DNA data, showed 2 separate lineages, in coincidence with their geographic distribution along riverbanks (Casado et al. 2007).

In this paper, we have analyzed the molecular divergence of *C. cay* across an extensive geographic region of South America comprising 2 different morphoclimatic domains (Cerrado in Brazil and Chaco of Southern Paraguay). This study indicated that these 2 populations probably split between 1.6 and 1.2 million years before present (MYBP).

## Material and Methods

Blood samples were collected from 23 wild-caught specimens of *C. cay* from Manso Hydroelectric dam reservoir, in Chapada dos Guimarães, Mato Grosso state, Brazil, along the rivers Manso and Quilombo and from 9 wild-caught

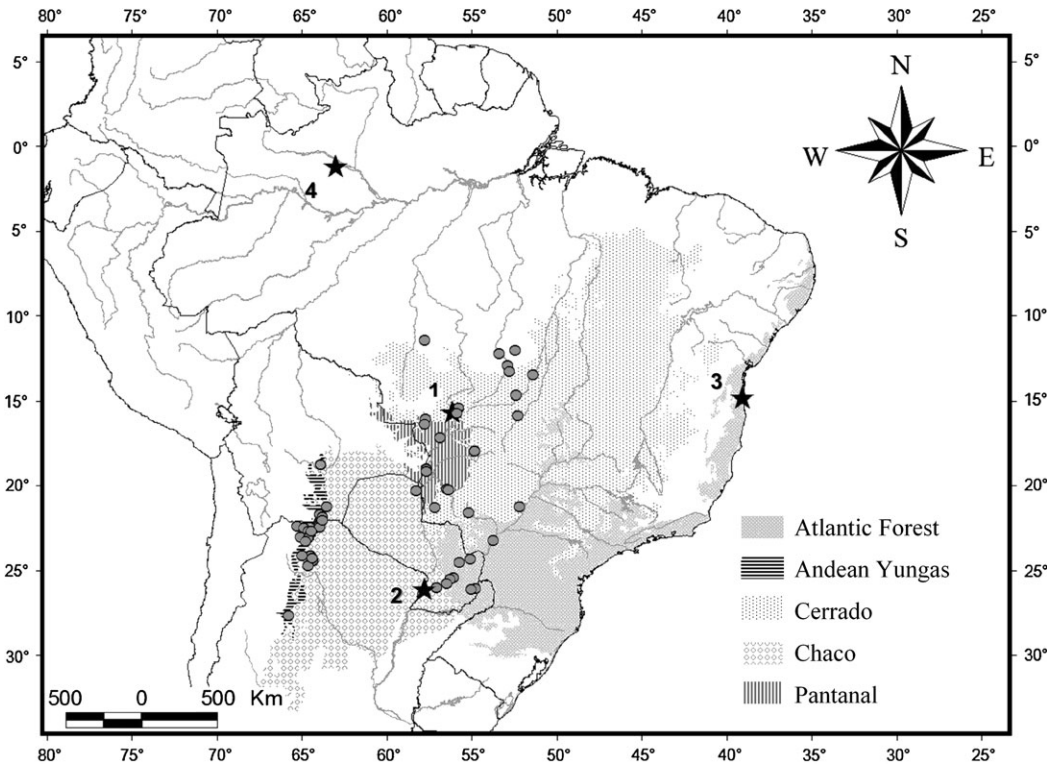


**Figure 1.** Geographic distribution of *Cebus cay*. Circles correspond to localities of *C. cay* reported by Silva Jr (2001). Stars indicate sites of *Cebus* herein analyzed: 1) *C. cay* from Mato Grosso state, Brazil; 2) *C. cay* from Santa Catalina province, Paraguay; 3) *Cebus xanthosternos* from Bahia state, Brazil; and 4) *C. apella*, *Cebus olivaceus* and *Cebus albifrons* from Amazonas state, Brazil.

**Table 1** List of analyzed *Cebus* specimens

| H | Specimen | Species | Sex | GenBank | Locality |
|---|---|---|---|---|---|
| 1 | CA549 | *Cebus cay* | F | FJ529046 | BRA, Mato Grosso, rio Quilombo |
| 1 | CA566 | *C. cay* | F | FJ529056 | BRA, Mato Grosso, rio Quilombo |
| 1 | CA644 | *C. cay* | n.a. | FJ529065 | BRA, Mato Grosso |
| 2 | CA553 | *C. cay* | M | FJ529047 | BRA, Mato Grosso, rio Quilombo |
| 3 | CA554 | *C. cay* | M | FJ529048 | BRA, Mato Grosso, rio Quilombo |
| 4 | CA555 | *C. cay* | F | FJ529049 | BRA, Mato Grosso, rio Quilombo |
| 5 | CA557 | *C. cay* | M | FJ529050 | BRA, Mato Grosso, rio Quilombo |
| 6 | CA559 | *C. cay* | M | FJ529051 | BRA, Mato Grosso, rio Quilombo |
| 7 | CA560 | *C. cay* | F | FJ529052 | BRA, Mato Grosso, rio Quilombo |
| 8 | CA562 | *C. cay* | F | FJ529053 | BRA, Mato Grosso, rio Quilombo |
| 9 | CA563 | *C. cay* | M | FJ529054 | BRA, Mato Grosso, rio Quilombo |
| 10 | CA565 | *C. cay* | M | FJ529055 | BRA, Mato Grosso, rio Quilombo |
| 11 | CA568 | *C. cay* | M | FJ529057 | BRA, Mato Grosso, rio Quilombo |
| 12 | CA614 | *C. cay* | F | FJ529058 | BRA, Mato Grosso, rio Quilombo |
| 13 | CA617 | *C. cay* | M | FJ529059 | BRA, Mato Grosso, rio Quilombo |
| 14 | CA619 | *C. cay* | n.a. | FJ529060 | BRA, Mato Grosso |
| 15 | CA627 | *C. cay* | F | FJ529061 | BRA, Mato Grosso, rio Quilombo |
| 15 | CA641 | *C. cay* | F | FJ529064 | BRA, Mato Grosso |
| 16 | CA628 | *C. cay* | F | FJ529062 | BRA, Mato Grosso |
| 17 | CA632 | *C. cay* | n.a. | FJ529063 | BRA, Mato Grosso |
| 18 | CA645 | *C. cay* | n.a. | FJ529066 | BRA, Mato Grosso |
| 19 | CA859 | *C. cay* | M | FJ529067 | BRA, Mato Grosso, rio Manso |
| 20 | CA865 | *C. cay* | F | FJ529068 | BRA, Mato Grosso, rio Manso |
| 21 | CAP7 | *C. cay* | M | FJ529069 | PAR, Santa Catalina |
| 22 | CAP10 | *C. cay* | F | FJ529070 | PAR, Santa Catalina |
| 23 | CAP14 | *C. cay* | F | FJ529071 | PAR, Santa Catalina |
| 24 | CAP55 | *C. cay* | M | FJ529072 | PAR, Santa Catalina |
| 24 | CAP59 | *C. cay* | F | FJ529073 | PAR, Santa Catalina |
| 24 | CAP66 | *C. cay* | F | FJ529074 | PAR, Santa Catalina |
| 24 | CAP68 | *C. cay* | F | FJ529075 | PAR, Santa Catalina |
| 24 | CAP76 | *C. cay* | F | FJ529076 | PAR, Santa Catalina |
| 24 | CAP475 | *C. cay* | M | FJ529101 | PAR, Santa Catalina |
| 25 | CRB2858 | *Cebus apella* | F | FJ529102 | BRA, Amazonas, Barcelos |
| 26 | CRB1806 | *C. apella* | F | FJ529103 | BRA, Amazonas, Barcelos |
| 26 | CRB2632 | *C. apella* | M | FJ529104 | BRA, Amazonas, Barcelos |
| 27 | 13190 | *Cebus xanthosternos* | n.a. | FJ529105 | BRA, Bahia |
| 28 | CRB2440 | *Cebus olivaceus* | M | FJ529106 | BRA, Amazonas, Barcelos |
| 29 | CRB2532 | *C. olivaceus* | M | FJ529107 | BRA, Amazonas, Barcelos |
| 30 | CRB1809 | *Cebus albifrons* | n.a. | FJ529108 | BRA, Amazonas, Barcelos |
| 31 | CRB2678 | *C. albifrons* | M | FJ529109 | BRA, Amazonas, Barcelos |

H, haplotype; n.a., not available; BRA, Brazil; PAR, Paraguay.

specimens from Santa Catalina region, northeastern Paraguay (Figure 1).

Sample collections were carried out following the national guidelines and provisions of Instituto Brasileiro do Meio Ambiente e Recursos Renováveis (Brazil) and the Ethics Committee of the Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno" (Argentina) in accordance with the principles and procedures described in the guidelines for the care and use of laboratory animals (Institute of Laboratory Animal Resources 1996).

DNA was isolated from all 32 *C. cay* from Mato Grosso state, Brazil and Santa Catalina, Paraguay, 3 *C. apella* from Amazonas state, one *C. xanthosternos* from Bahia state, and 2 *C. albifrons* and 2 *C. olivaceus* from Amazonas state, Brazil (Table 1) following Smith et al. (1987). The mitochondrial cytochrome *b* gene (*MT-CYB*) was amplified with primers L14724 (Irwin et al. 1991) and CIT-REV (5′-GAATATCAGCTTTGG-3′). The conditions for amplification were 200 mM of each nucleotide, 1.0 mM of each primer, 1.5 mM $MgCl_2$, 1.0 U of Platinum *Taq* polymerase (Invitrogen), 1× of reaction buffer, and 100 ng of DNA. The reaction started with denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min, and extension at 72 °C for 1 min and 30 s. Both DNA strands of purified, amplified products were sequenced following labeling with the same primers, with the DYEnamic ET Dye Terminator Sequencing kit, and run in MegaBace 1000 and ABI Prism 377 automatic sequencers. Sequences were manually aligned and edited using Bioedit 7.0.5 (Hall 1999) and Chromas 1.45 softwares (McCarthy 1996–1998). Cytochrome *b* nomenclature follows HGNC rules (HGNC 2008). Table 1 lists all studied specimens, haplotypes, GenBank accession numbers, sex, and locality.

Genetic distance estimates, maximum parsimony (MP), maximum likelihood (ML), and neighbor joining (NJ) analyses were carried out with single haplotypes. "Genetic distance and NJ were estimated with the p-distance model with pairwise deletion using the Molecular Evolutionary Genetics Analyses software (MEGA 4; Tamura et al. 2007). The p-distance model was chosen because the number of nucleotide substitutions per site was 0.03. According Nei and Kumar (2000), the p distance or the Jukes–Cantor distance is recommended when the number of substitutions per site is equal or below 0.05.

Phylogenetic analyses were carried out with PAUP* 4.0b10 (Swofford 2003) for MP and ML. Construction of the MP and ML topologies was carried out by heuristic search with stepwise addition of branches with 10 random replicates implemented by the algorithm tree bisection reconnection. ML analysis was carried out with the best-fit model of evolution of base substitution selected by alkaline information criterion test with MODELTEST 3.07 (Posada and Crandall 1998), GTR+G+I (general time reversible + Gamma + proportion invariant) model with estimation of invariable sites (0.50) and gamma correction (0.95). The ratchet strategy (Nixon 1999; Vos 2003) was used for MP and ML. ML analyses were also carried with partitioned data (by codon position). The consistency of nodes was estimated with 1000 bootstrap replicates for MP and NJ, and 500 for ML (Felsenstein 1985). Trees were rooted using species of the subgenus *Cebus*.

The Arlequin 3.11 software (Excoffier and Schneider 2005) was used for estimating the intra- and interpopulation variance (Analysis of molecular variance [AMOVA]; Excoffier et al. 1992) and mismatch distribution (Schneider and Excoffier 1999). In the former analysis, genetic divergence was calculated between *C. cay* from Brazil and Paraguay and between Brazilian *C. cay* subgroups according to locality of capture: rio Quilombo, rio Manso, and undetermined (see Table 1). Conversely, the latter analysis assumes population expansion as a null hypothesis and generates a theoretical distribution of differences among haplotypes that is compared with the real distribution of data. *Cebus cay* from Brazil and Paraguay were thus grouped for this analysis in view of the small number of individuals from Paraguay. We estimated parameter $\tau$, as an estimate of the time of occurrence of the hypothetical expansion, where $\tau = 2\mu t$; $t$ = time, expressed in number of generations since the population expanded; $\mu$ = mutation rate per generation for the segment of DNA under study, and $\theta$ as estimate of population size ($\theta = 2N\mu$ where $N$ = effective size of the population's females; Schneider and Excoffier 1999). In order to justify the assumption of mutation-drift equilibrium in mismatch distribution analysis we used neutrality test of Fu (1997) ($F_s$ statistic), with Arlequin 3.11. Significance was estimated using 1000 permutations.

Median joining (MJ) analysis was carried out with Network 4.5.0.0 (Bandelt et al. 1999; http://www.fluxus-engineering.com) for analyzing intraspecific phylogenies and evaluating structure and geographic distribution patterns of *C. cay*. We used *C. apella* as outgroup in the *C. cay* MJ network, similarly to analyses of species exhibiting very low interspecific divergence (Gonzalez-Ittig et al. 2007).

The Beast package v1.4.8 (Drummond et al. 2006), using a Bayesian Markov chain Monte Carlo (MCMC) algorithm, was used for estimating the time of the most recent common ancestor (TMRCA) of *C. cay* from Brazil and Paraguay. To improve the accuracy of our estimates, we used one *Saimiri sciureus* (AJ489756) and one *Aotus trivirgatus* (DQ098874) as outgroups. The input file was generated assuming an uncorrelated relaxed lognormal clock for sequence evolution, a GTR+G+I substitution model identified by MODELTEST, and a Yule process for model speciation as the tree prior. To calibrate our analysis, we assumed a normal TMRCA distribution, allowing divergence dates to vary symmetrically. The rate of divergence of cytochrome *b* was calibrated at 22.0 MYBP corresponding to the splitting of the Cebidae (sensu Goodman et al. 1998; Schneider et al. 2001) and a range of 17.0–20.0 MYBP for the divergence of *Cebus/Saimiri* (Opazo et al. 2006), with a normally distributed calibration mean of 19.0 and a standard deviation of 1.0 (credibility intervals: 5% = 17.36; 95% = 20.64). Each final MCMC chain was run for 50 000 000 generations (burn-in = 10%), with parameters samples every 1000 steps. Results of runs were displayed in Tracer v1.4.1 (from BEAST package) to check for stationary distribution.

## Results

All polymerase chain reaction amplified cytochrome *b* products showed a single band, and all electropherograms were clear, without ambiguities, showing of a single open reading frame encompassing the complete gene in all specimens, with a deduced protein sequence of 380 aminoacids. These findings suggested that amplified products corresponded to true *MT-CYB* DNA rather than numts (nuclear mitochondrial DNA) (Zhang and Hewitt 1996; Sorenson and Quinn 1998; Bensasson et al. 2001; Nascimento et al. 2008).

Cytochrome *b* DNA, comprising 1140 base pairs, showed 24 different haplotypes in 32 specimens of *C. cay*; 4 haplotypes being present in more than one individual (Table 1). None of these haplotypes were shared between Brazilian and Paraguayan *C. cay*.

We found 29 variable sites along the entire *C. cay* gene, with 19 transitions and 10 transversions; most replacements occurring at third codon position (40.0%) contrary to first and second positions (26.7% and 33.0%, respectively).

Genetic distance estimates between *C. cay* haplotypes varied from 0.1% to 1.0%, with an average of 0.5% (±0.4). Genetic variation in the sample from Mato Grosso (Brazil) showed an average of 0.4% (±0.2) ranging between 0.1 and 1.0 and Santa Catalina (Paraguay) showed an average of 0.3% ± 0.1% ranging between 0.1 and 0.4. However, when these groups were compared, distance estimates showed an average of 1.1% (±0.2). Similar results were found between *C. cay* and *C. apella*, with an average genetic distance of 1.3% (±0.2).

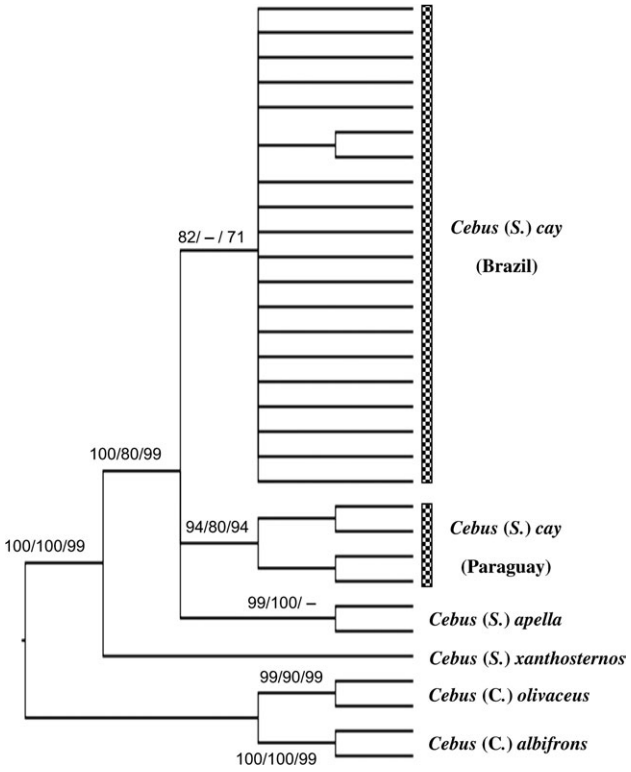


**Figure 2.** Consensus tree with MP analysis. Numbers above branches indicate bootstrap values from parsimony, likelihood, and NJ analyses, respectively. Consistency index = 0.8525; retention index = 0.9149; rescaled consistency index = 0.7799; – = bootstrap estimate below 50.

MP, ML, and NJ analyses showed the monophyly of the *Cebus* taxa herein studied and of both subgenera (*Cebus* and *Sapajus*). MP and ML showed a trichotomy formed by 2 *C. cay* branches and *C. apella*; ML analyses with partitioned data (by codon position) failed to demonstrate the monophyly of *C. cay*. NJ analysis grouped *C. cay* and *C. apella* as sister groups, and in all analyses, *C. xanthosternos* was identified as a sister group of all species of the subgenus *Sapajus* (Figure 2). All topologies were congruent in separating Paraguayan from Brazilian haplotypes (see also Figure 3).

AMOVA showed φ estimates with significant differences ($P < 0.05$) for the correlation of random haplotypes, with the highest variance component between *C. cay* from Brazil and Paraguay (62.9%), indicating that these populations were genetically divergent (Table 2).

Mismatch distribution for *C. cay* haplotypes showed a unimodal curve, similarly to the simulated curve. The graph showed nonsignificant *P* values (>0.05; Figure 4), indicating that the simulated curve did not differ from the observed curve, suggesting a recent demographic expansion. Additionally, a large difference was observed between the initial ($\theta_0 = 4.37$) and the final ($\theta_1 = 99999.00$) estimates of effective population size. Moreover, the $F_s$ statistical value was strongly negative for *C. cay* (−21.564; $P < 0.01$), supporting the hypothesis that this species has undergone a substantial growth.

MJ analysis showed 39 variable sites (Figure 5). This analysis separated *C. cay* haplotypes from Brazil apart from *C. cay* from Paraguay by at least 8 mutational steps and one median vector, and both of them from the *C. apella* outgroup by at least 7 mutations steps and one median vector. The most frequent haplotypes of the 2 *C. cay* groups were interconnected by a same and single median vector with the outgroup indicating that they might be relatively older with respect to the others (Avise et al. 1987; Crandall and Templeton 1993).

We estimated coalescence times for major nodes with a mutation rate of 1–2% per MYBP. A calibrated ML tree estimated the TMRCA for *C. cay* from Brazil and Paraguay at 2.0 MYPB, *C. apella* from *C. cay* at 2.6 MYPB, and the 2 subgenera (*Cebus* and *Sapajus*) at 4.2 MYPB (Figure 3). These results, inferred from the patterns of haplotype diversity, strongly suggested a very recent diversification of *C. cay* during the Early Pleistocene.

## Discussion

We found 20 haplotypes in 23 *C. cay* from Brazil but only 4 haplotypes in 9 *C. cay* from Paraguay. Genetic distance estimates showed that Brazilian and Paraguayan *C. cay* differed by 1.1%, and a similar estimate was found between pooled *C. cay* haplotypes and *C. apella* (1.3%). This divergence was higher than distance estimates of *MT-CYB* in other neotropical primates like *Alouatta*, with 0.7% between *Alouatta macconnelli* and *Alouatta nigerrima* (Bonvicino et al. 2001), but lower than other intrageneric distances, as found in *Callicebus*, with 4.0% between *C. lugens* and *Callicebus torquatus* (Bonvicino et al. 2003; Casado et al. 2007), and 6.0% between *Callicebus nigrifrons* and *Callicebus personatus* (Bonvicino et al. 2003). In *Alouatta*, *Alouatta pigra* and *Alouatta palliata* were found to diverge by 4.0% (Nascimento et al. 2005), and in *Saguinus*, Cropp et al. (1999) reported an estimate of 3.4% between *Saguinus midas* and *S. niger*

**Table 2** Intra- and interpopulation variance estimates between groups of *Cebus cay* (AMOVA)

| | Pairwise difference | | | | |
|---|---|---|---|---|---|
| Source of variation | Degrees of freedom | Sum of squares | Variance | % of variation | Fixation indices |
| Between *C. cay* from Brazil and Paraguay | 1 | 27.8 | 3.788 | 62.9 | $\varphi_{CT} = 0.629$ |
| Between *C. cay* groups of different regions of capture in Brazil | 2 | 5.0 | 0.073 | 1.2 | $\varphi_{SC} = 0.033$ |
| Between *C. cay* groups in Brazil and *C. cay* of Paraguay | 20 | 43.3 | 2.163 | 35.9 | $\varphi_{ST} = 0.641$ |

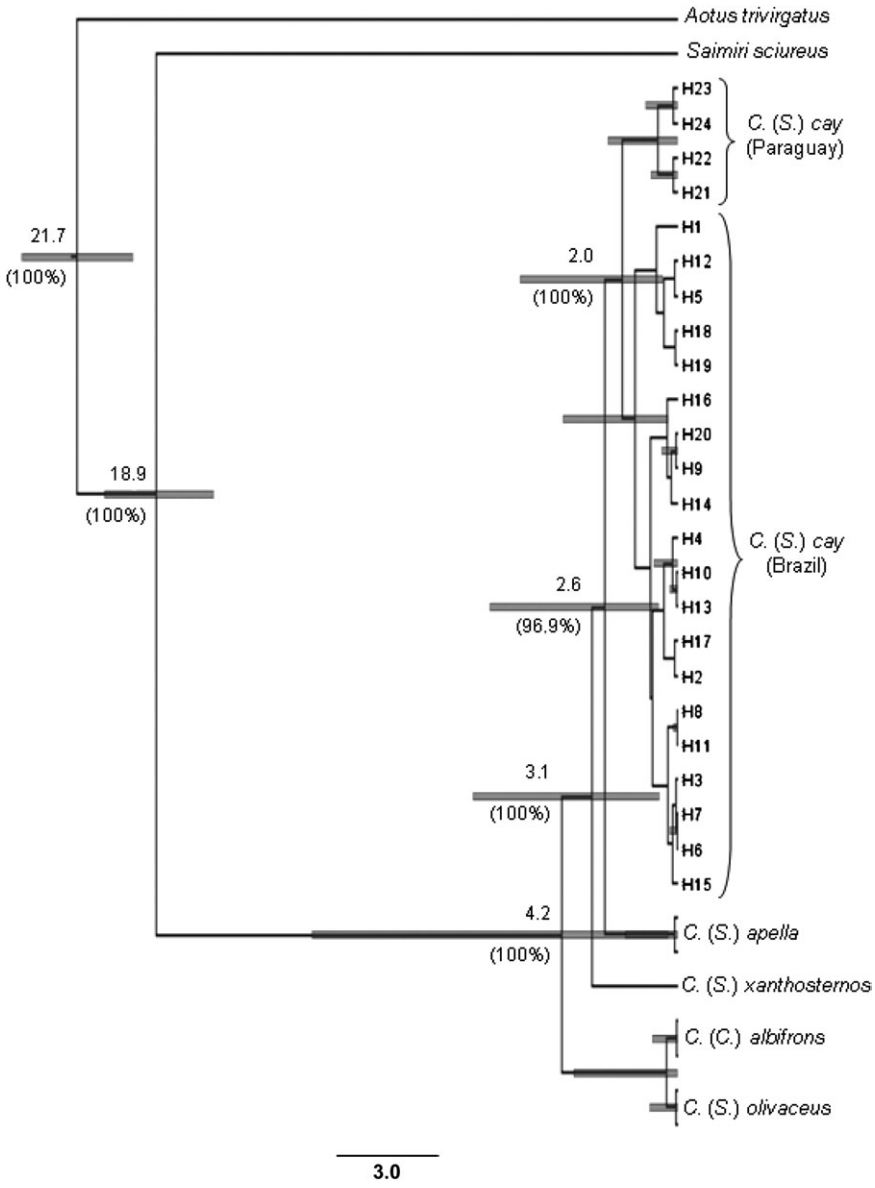


**Figure 3.** Divergence time estimates based on the maximum likelihood tree topology. Grey bars represent 95% HPD (highest posterior density) interval for divergence time estimates. Numbers above nodes indicate time of divergence in MYBP, and numbers in parentheses indicate posterior probabilities. H = haplotype (see Table 1).

and 4.1% between *Saguinus bicolor* and *Saguinus martinsi*. On the other hand, intraspecific divergence estimates in *Alouatta belzebul* (up to 1.2%; Nascimento et al. 2007, 2008) were similar in *C. cay* (1.1%) but lower than in *C. lugens* and *S. mystax* (2.1%; Cropp et al. 1999; Casado et al. 2007). These findings suggested that the separation between *C. cay* and *C. apella* was likely to be recent and not indicative of a substantial divergence between these taxa.

Lack of definition for solving the relationship between *C. cay* and *C. apella* in MP and ML topologies must result from the low level of genetic divergence between them. This was also shown by mismatch distribution analysis, indicating that *C. cay* population expansions must have been very recent, having diverged in a very short time span from one another, during which very few synapomorphies could have emerged. Lack of shared haplotypes between Brazilian and Paraguayan *C. cay* and the structure of these populations shown by AMOVA and MJ Network indicated a genetic divergence pattern corresponding to categories I or III of Avise et al. (1987). The MJ network (Figure 5), however, rather corresponded to category I of Avise, of "phylogenetic discontinuities, spatial separation," a category that includes populations separated for a long time by zoogeographic barriers, accumulating mutations resulting in higher genetic divergence between groups than within groups, and connected by the most frequently shared haplotypes in each

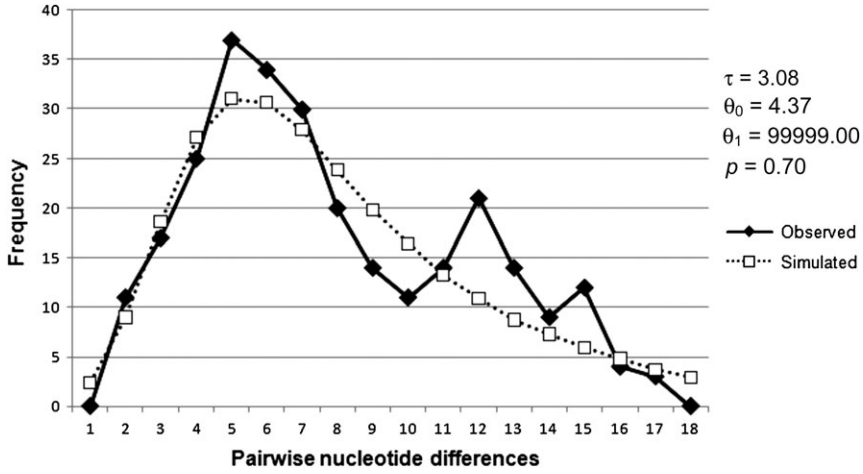


**Figure 4.** Mismatch distribution of *Cebus cay*. $\tau$ = estimator of time elapsed since expansion; $\theta_0$ = estimator of initial population size; $\theta_1$ = estimator of population size after expansion.

population. However, despite the evident separation of Brazilian from Paraguayan *C. cay*, category III (phylogenetic continuity, spatial separation) cannot be dismissed in view of the lack of collected samples between Southern Paraguay and western central Brazil. This latter category comprises geographically separated populations still showing a phylogenetic continuity, with a recent interruption of gene flow, and with individuals still sharing plesiomorphic characteristics.

Our divergence time estimates between the 2 populations of *C. cay*, suggested that they probably split in the Early Pleistocene. This period is characterized by repeated glaciation events, leading to drastic changes in the vegetation composition within different biomes (Vivo and Carmignotto 2004). Some authors postulated that evergreen forests receded during the glacial period and consequently leaving open space for the emergence of open savannas. However,

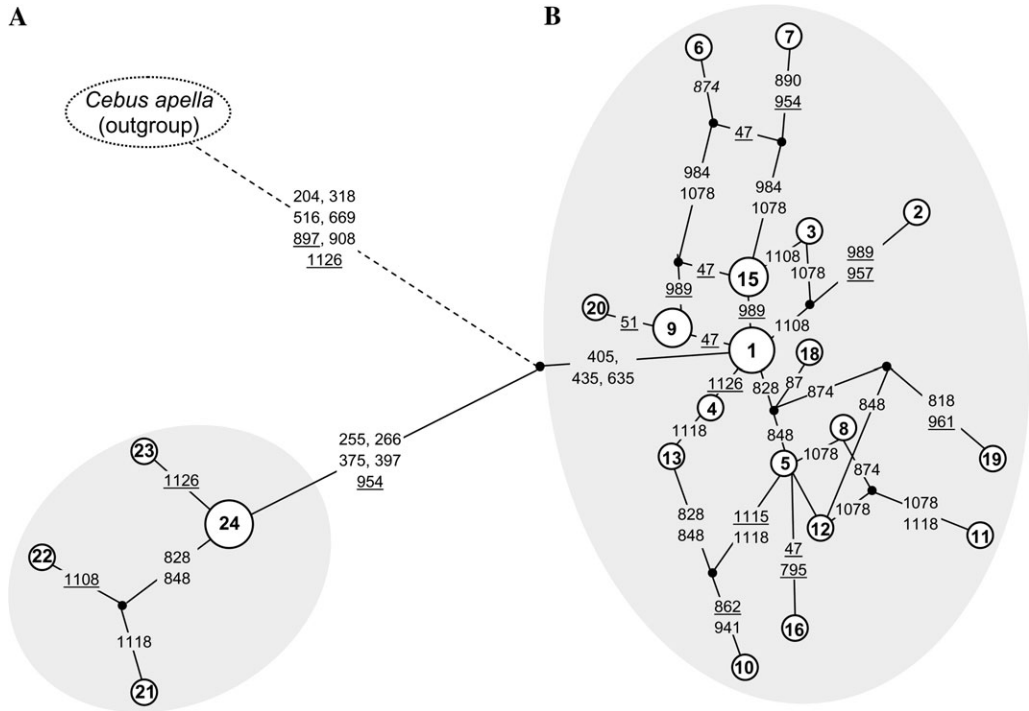


**Figure 5.** MJ Network of *Cebus cay* from Paraguay (**A**) and Brazil (**B**). Numbers inside circles indicate haplotypes listed in Table 1; size of circles is proportional to the number of specimens sharing a given haplotype. Black circles are median vectors. Numbers in connecting branches denote nucleotide substitutions (transitions = not underlined; transversions = underlined).

when climatic conditions changed in the Holocene, open formations were eliminated in some areas or were reduced in others, being substituted by dense savanna, evergreen and semi evergreen forests (Bradbury et al. 1981; Bigarella and Andrade-Lima 1982; Hare 1992; de Oliveira et al. 1999; Joly et al. 1999; Vivo and Carmignotto 2004). Eventually, these physiognomies changed gradually. The savanna became less dense (Cerrado) and part of the evergreen and semi evergreen forests were reduced and substituted by dry forest (Chaco). Probably, in these current vegetation physiognomies, species groups adapted to forests became isolated to restricted regions (Vivo and Carmignotto 2004). This scenario may explain the evident discontinuity between the *C. cay* populations of western central Brazil and northeastern Paraguay from those of northwestern Argentina and southern Bolivia resulting from their separation by the Chaco and the separation between Paraguayan and Brazilian populations by open vegetation formations of Cerrado and arid Chaco areas.

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