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ORIGINAL ARTICLE

Molecular phylogenetics and phylogeography of all the *Saimiri* taxa (Cebidae, Primates) inferred from *mt COI* and *COII* gene sequences

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Abstract Some previous genetic studies have been performed to resolve the molecular phylogenetics of the squirrel monkeys (*Saimiri*). However, these studies did not show consensus in how many taxa are within this genus and what the relationships among them are. For this reason, we sequenced 2,237 base pairs of the *mt COI* and *COII* genes in 218 *Saimiri* individuals. All, less 12 *S. sciureus sciureus* from French Guyana, were sampled in the wild. These samples represented all the living *Saimiri* taxa recognized. There were four main findings of this study. (1) Our analysis detected 17 different *Saimiri* groups: *albigena*, *cassiquiarensis*, five polyphyletic *macrodon* groups, three polyphyletic *ustus* groups, *sciureus*, *collinsi*, *boliviensis*, *peruviensis*, *vanzolinii*, *oerstedii* and *citrinellus.* Four different phylogenetic trees showed the Central American squirrel monkey (*S. oerstedii*) as the most differentiated taxon. In contrast, *albigena* was indicated to be the most recent taxon. (2) There was extensive hybridization and/or historical introgression among *albigena*, different *macrodon* groups, *peruviensis, sciureus* and *collinsi*. (3) Different tests showed that our maximum likelihood tree was consistent with two species of *Saimiri*: *S. oerstedii* and *S. sciureus*. If no cases of hybridization were detected implicating *S. vanzolinii*, this could be a third recognized species. (4) We also estimated that the first temporal splits within this genus occurred around 1.4–1.6 million years ago, which indicates that the temporal split events within *Saimiri* were correlated with Pleistocene climatic changes. If the biological species concept is applied because, in this case, it is operative due to observed hybridization in the wild, the number of species within this genus is probably more limited than recently proposed by other authors. The Pleistocene was the fundamental epoch when the mitochondrial *Saimiri* diversification process occurred.

Keywords *Saimiri* · Phylogenetics and phylogeography · Mitochondrial *COI* and *COII* genes · Biological species concept · Pleistocene climatic changes

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Introduction

Members of the *Saimiri* genus (squirrel monkeys) are small-sized Neotropical monkeys. They weigh approximately 700–1,000 g and are very agile, quadruped and arboreal (Robinson and Janson 1987), having a wide distribution in Colombia, Venezuela, Guyana, Suriname, French Guyana, Ecuador, Peru, Bolivia and Brazil, with some additional isolated populations in Costa Rica and Panama.

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Many morphological classifications have been proposed for the *Saimiri* genus since the beginning of the 20th century and have ranged from the inclusion of one to seven species and up to 17 different subspecies (Elliot 1913, Lönnberg 1940, Cabrera and Yepes 1940, von Pusch 1942 and Hill 1960). Cabrera and Yepes (1940) recognized three species (*S. sciureus*, *S. ustus*, and *S. boliviensis*). However, Cabrera (1957), Napier and Napier (1967), Cooper (1968), Hershkovitz (1972), Napier (1976), Coimbra-Filho and Mittermeier (1981) considered the existence of only one or two species (*S. sciureus* or *S. sciureus* and *S. oerstedii*). Thorington (1976) also suggested that there was only a single *Saimiri* species because he noted that there was a cline of intermediate forms between the Colombian (*sciureus*) and Bolivian (*boliviensis*) squirrel monkeys. Thorington (1976) also concluded that the most differentiated form of *Saimiri* was from the Madeira River (*madeirae*), but that it was a subspecies of *S. sciureus*.

However, Hershkovitz (1984), in his review, proposed the existence of two species groups, four species with a total of nine taxa. He based this proposal on his analysis of behavioral patterns, geographic distribution and morphologic patterns such as coat color, the morphology of eyebrows and tail color. His two species groups were the Roman type, containing *S. b. boliviensis* and *S. b. peruviensis* and the Gothic type, containing *S. sciureus* (with four subspecies: *S. s. sciureus*, *S. s. macrodon*, *S. s. albigena* and *S. s. cassiquiarensis*), *S. oerstedii* (with two subspecies: *S. o. oerstedii* and *S. o. citrinellus*) and *S. ustus* (synonymous with *S. madeirae*).

The geographic distributions of the taxa defined by Hershkovitz (1984) were as follows: (1) *S. b. boliviensis* is distributed south of the Jurua and Amazon rivers and is in the majority of the Purus, Mamore, Beni, Madre de Dios and Guapore watersheds in Peru, Brazil and Bolivia. (2) *S. b. peruviensis* is distributed in the Peruvian Amazon at the Ucayali River. (3) *S. sciureus sciureus* is distributed in the north-east section of the Brazilian Amazon (east of the Negro River), including the Guyanas, to the Amazon's mouth. (4) *S. sciureus albigena* is an endemic taxon from Colombia, whose distribution extends from the Eastern Andes to the Vichada Department, near the frontier with Venezuela. There is another population west of the Eastern Andes in the upper Magdalena River basin (Cauca and Huila Departments). (5) *S. sciureus cassiquiarensis* is distributed in the Colombian Amazon north of the Apaporis River to the west side of the Negro River in the northern Brazilian Amazon. (6) *S. sciureus macrodon* has a geographical distribution that ranges south of the Apaporis River in the Colombian Amazon, the Ecuadorian Amazon, the northern part of the Peruvian Amazon and the western Brazilian Amazon. (7) *S. ustus* distribution extends from the right bank of the Tefe Lake to the Xingu and Iriri rivers in the south-central area of the Brazilian Amazon. (8) *S. oerstedii oerstedii* has a limited distribution on the Pacific coast of southern Costa Rica (Puntarenas) and northern Panama (Chiriquí and Veraguas). (9) *S. oerstedii citrinellus* has a very restricted distribution in the middle Pacific coast of Costa Rica in Manuel Antonio National Park.

The independent classification proposed by Thorington (1985) is based on patterns of coloration and on morphometric analyses of skulls and teeth. He recognized two species and five subspecies. The first species was *S. sciureus* — including four subspecies: *S. s. sciureus*, *S. s. boliviensis*, *S. s. cassiquiarensis* and *S. s. oerstedii*. The second species was *Saimiri madeirae*.

Another species, *S. vanzolinii*, was described by Ayres (1985), subsequent to the classifications proposed by Hershkovitz (1984) and Thorington (1985). The description is based on morphological features and the geographic distribution, restricted to 950 km^2 in the confluence of the Japura and Amazon rivers, in the Brazilian Amazon. The species belongs to the Roman group.

Hershkovitz (1987) commented that two more *boliviensis* subspecies could exist, *S. boliviensis pluvialis* and *S. boliviensis jaburuensis* and that the new taxon proposed by Ayres (1985) could be another subspecies of *S. boliviensis*, *S. boliviensis vanzolinii*. Thus, globally, Hershkovitz defined four species and 12 taxa.

Later, Costello et al. (1993) analyzed some genetic and behavioral data and determined possible hybridization between *S. sciureus*, *S. ustus* and *S. boliviensis.* Therefore, they only accepted two species, *S. sciureus* (for all the South American taxa) and *S. oerstedii* (for the isolated Central American form).

Groves (2001) recognized the two groups described by Hershkovitz (1984). Within the Roman arch group, he recognized *S. boliviensis boliviensis*, *S. b. peruviensis* and *S. vanzolinii,* whereas within the Gothic group he included, *S. oerstedti oerstedti*, *S. oerstedti citrinellus*, *S. sciureus sciureus*, *S. sciureus albigena*, *S. sciureus cassiquiarensis*, *S. sciureus macrodon* and *S. ustus*. Therefore, although the Groves book (2001) radically changed the classifications of some primates, for *Saimiri* he basically agreed with the Hershkovitz (1984) classification.

To date, no molecular studies have been conducted with all the *Saimiri* taxa suggested by the classification of Groves (2001), nor with large sample sizes. The first two molecular phylogenetic works with *Saimiri* were those of Boinski and Cropp (1999) (14 *Saimiri* samples) and Cropp and Boinski (2000) (11 *Saimiri* samples). They determined four major clades: (1) *S. oerstedii* with the two subspecies *S. o. oerstedii* and *S. o. citrinellus*; (2) *S. boliviensis* with the two subspecies *S. b. boliviensis* and *S. b. peruviensis*; (3) *S. ustus* and (4) *S. sciureus*. The divergence time between *S. sciureus* and *S. boliviensis* was estimated to

have occurred at 1.14–6.42 million years ago (MYA). Later, Lavergne et al. (2010) analyzed 32 samples of *Saimiri* for the *mt Cyt-b* gene. They showed that the main temporal split within *Saimiri* was around 4.3 MYA and that the *sciureus* complex was not monophyletic. The last study was that of Chiou et al. (2011). They sequenced 12 *mt* genes with around 10,800 bp of seven *Saimiri* individuals plus two mitochondrial genomes obtained from Genbank (one captive animal and another with an unknown origin). Their main results indicated that the ancestors of *boliviensis* were the first to diverge and that the divergence times were considerably lower than those obtained by Cropp and Boinski (2000) and Lavergne et al. (2010). The initial temporal split within *Saimiri* was estimated by these authors to be around 1.5 MYA and the major part of the splits was around 0.9–1.1 MYA.

Therefore, this is the fifth molecular phylogenetic study with *Saimiri*. In this work, we present, to our knowledge, the first attempt to resolve the phylogeny and phylogeography of all the *Saimiri* taxa by using the mitochondrial cytochrome oxidase subunit I and II genes (*mt COI* and *COII*) from the largest wild-living sample obtained to date (218 individuals), representing all the recognized species and subspecies of this genus accepted by Groves (2001). All of these samples, with the exception of 12 samples of *S. sciureus sciureus* from French Guyana, were obtained from the wild. This is very important because animals from captive colonies can have unknown origins, can be erroneously classified and can be directly or indirectly descended from hybridization in captivity. We even added some samples of a possible different taxon not considered by Groves (2001). Osgood (1916) reported that *S. s. collinsi*, found on Marajo Island in the Amazon's mouth, has similar color patterns to that of *S. s. sciureus* in Guyana but with some small morphological differences. In fact, this taxon was recognized by da Cruz Lima (1945), Cabrera (1957) and Hill (1960), but considered a synonym of *S. s. sciureus* by Hershkovitz (1984) and Groves (2001).

The mitochondrial genes are interesting markers for phylogenetic tasks because they include a rapid accumulation of mutations, lack introns, have a high number of copies per cell, a negligible recombination rate and include haploid inheritance (Avise et al. 1987). The selected genes are two of the three mitochondrial DNA encoded subunits (*COI*, *COII* and *COIII*) of respiratory complex IV. *Mt COI* has emerged as the standard barcode region for animals, including mammals (www.mammaliabol.org). Hebert et al. 2003a, b; 2004a, b) have strongly argued in favor of using a 5′ fragment of this mitochondrial gene as a barcoding marker that has shown to provide a sufficient resolution and robustness in some groups of organisms, such as arthropods, fishes, birds and mammals, to distinguish species (Agrizzi et al. 2012; Lim 2012).

The second gene has been studied extensively in primate phylogenetics (Adkins and Honeycutt 1991; Ruvolo et al. 1991; Ruiz-García et al. 2010; 2012a, b).

Taking all of these facts into account, the main aims of the current work with *Saimiri* were twofold: firstly, to determine the phylogenetic relationships among 218 *Saimiri* individuals, sampled in the wild, and representing all the morphological taxa recognized by Groves (2001) and to compare these new molecular results with the morphological classifications and previous molecular systematics of *Saimiri;* and secondly, to determine possible hybridization or introgression events among different *Saimiri* taxa.

Methods

Samples

A total of 218 squirrel monkeys were sampled in the wild representing all the morphological *Saimiri* taxa recognized. These 218 samples were classified "a priori", based on the morphological characteristics and their geographical origins. These samples were classified as follows: (1) A total of 119 *albigena,* which were sampled in different Colombian Departments: 88 from the Meta Department, 12 from the Casanare Department, 5 from the Huila Department, 11 from the Boyaca Department and 3 from the Arauca Department. (2) A total of 14 *cassiquiarensis* specimens were sampled in Colombia, 3 from diverse points of the Guaviare River, 4 from the Guainia River, 3 from the Inirida River and 4 from the Vaupes River. (3) A total of 36 *macrodon* specimens were sampled in Colombia, Peru and Ecuador: in Colombia, 19 from the Putumayo, Caqueta and Amazonas Departments; in Peru, 13 from the Loreto and Ucayali Departments and 4 in Ecuador. (4) Ten *ustus* specimens from Brazil: two from the Purus River, five from the Amazon River and three from the Madeira River. (5) A total of 13 *sciureus* individuals were sampled; 12 from the Pasteur Institute colony from French Guyana and 1 from Balbina, northern Brazilian Amazon. (6) Five *collinsi* exemplars were sampled in the Marajo Island at the Amazon mouth in Brazil. (7) Three *boliviensis* individuals were sampled at the Santa Cruz Department in Bolivia. (8) A total of 14 *peruviensis* specimens were sampled in Peru: 1 in Parinari, on1e at the Samiria River, 3 at the Marañon River, 6 at the Ucayali River, 1 at Quistococha and 2 at the Amazon River (Loreto and Ucayali Departments). (9) One *vanzolinii* exemplar sampled at the Mamiraua Lake in central Amazon in Brazil. (10) One *oerstedii* individual sampled at the Chiriqui River in Panama and (11) Two *citrinellus* individuals sampled at the Manuel Antonio National Park in Costa Rica. For more details see Fig. 1 and Appendix 1.

Fig. 1 Map with the geographical distributions of the 17 different *Saimiri* groups recognized in this work and with the geographical areas where different *Saimiri* taxa were sampled

Seven *Cebus albifrons* were used as outgroups [from the Napo River-Peru (1), Ucayali River-Peru (1), Negro River-Brazil (1), Leticia at the Amazon River-Colombia (3) and Antioquia-Colombia (1)].

The DNA was extracted from hairs obtained from animals found alive in diverse Indian and colonos communities throughout Panama, Colombia, Peru, Ecuador, Brazil and Bolivia. The DNA obtained from animals of Costa Rica and French Guyana was from blood samples. We also requested permission to collect biological materials from dead animals that were already present in the communities sampled. In these cases, we sampled small pieces of muscles or teeth from hunted animals. Communities were visited only once, all sample donations were voluntary, and no financial or other incentive was offered for supplying specimens for analysis. All the pets and the hunted animals analyzed were obtained by the Indian communities at a maximum of 20 km from their community. For more information about sample permissions, see the Acknowledgment section.

Molecular analyses

Mt COI and COII genes

The DNA from muscle and blood was extracted using the phenol–chloroform procedure (Sambrook et al. 1989), while DNA samples from hair and teeth were extracted with 10 % Chelex resin (Walsh et al. 1991). For the *mt COI* amplification (polymerase chain reaction, PCR), we used the forward primer LCO5355 (5′-GGTCAACAAATCAT AAAGATATTGG-3′) and the reverse primer HCO6899 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (1,544 base pairs, bp) (Folmer et al. 1994) under the following PCR profile: 94 °C for 5 min, followed by 39 cycles of 94 °C for 30 s, 44 °C for 45 s, 72 °C for 45 s and a final cycle of 72 °C for 5 min. For the amplification of the *mt COII* gene (located in the lysine and asparagine tRNAs) we used the primers L6955 (5′-AACCATTTCATAACT TTGTCAA-3′) and H7766 (5′-CTCTTAATCTTTAACTT AAAAG-3′) (693 bp) (Collins and Dubach 2000;

Ruiz-García et al. 2010; 2012a, b. We used the following temperatures: 95 °C for 5 min, 35 cycles of 45 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C and a final extension time for 5 min at 72 °C. For both genes, the PCRs were performed in a 25-µl volume with reaction mixtures including 2.5 µl of 10× buffer, 6 µl of 3 mM $MgCl_2$, 2 µl of 1 mM dNTPs, 2 µl (8 pmol) of each primer, 2 units of Taq DNA polymerase, 6.5 µl of H_2O and 2 µl (20–80 ng/µl) of DNA. PCR reactions were carried out in a BioRad thermocycler. All amplifications, including positive and negative controls, were checked in 2 % agarose gels, using the molecular weight marker ϕ 174 DNA digested with *Hind* III and *Hinf* I. The amplified samples were purified using membrane-binding spin columns (Qiagen). The double-stranded DNA was directly sequenced in a 377A (ABI) automated DNA sequencer. The samples were sequenced in both directions using the BigDye ™ kit, and all the samples were repeated to ensure sequence accuracy. As the population genetics and phylogenetic analyses of both mitochondrial genes support similar findings, we only highlight the results of when both genes were analyzed together (1,544 + 693 = 2,237 bp). The sequences obtained were deposited in GenBank (accession numbers: KF356028 to KF356245).

Data analyses

Phylogenetic analyses

The sequence alignments were carried out manually and with the DNA alignment program (Fluxus Technology Ltd.). The Modeltest software (Posada and Crandall 1998) and the Mega 5.1 software (Tamura et al. 2011) were applied to determine the best evolutionary mutation model for the 225 sequences analyzed for both gene sequences concatenated. Akaike information criteria (AIC; Akaike 1974) were employed to determine the best evolutionary mutation model.

We obtained maximum likelihood estimates of transition/transversion bias as well as maximum likelihood estimates of gamma parameter for site rates for the best evolutionary mutation model obtained (Tamura et al. 2011).

The phylogenetic trees were obtained by means of four different procedures for the individual genes and for both genes concatenated: (1) Neighbor-joining (NJ; Saitou and Nei 1987) tree with the Kimura 2P genetic distance (Kimura 1980). (2) Minimum evolution (ME; Li 1997) tree with the maximum compositum distance (Nei and Kumar 2000). (3) Maximum likelihood (ML; Felsenstein 1981) tree with the GTR model. All these trees were constructed with the PAUP*4.0b8 program (Swofford 2002) and MEGA 5.1. (4) A Bayesian tree (BT; Mau 1996; Mau et al. 1999; Rannala and Yang 1996) was performed using the GTR model of nucleotide substitution with the gamma distributed rate varying among sites, and 11 rate categories (GTR + G) because it was determined to be the better model using the Modeltest program. This Bayesian analysis was completed with the BEAST v1.6.2 program (Drummond and Rambaut 2007). Two separate sets of analyses were run, assuming a Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution (Drummond et al. 2006). Results from the two independent runs (40,000,000 generations with the first 4,000,000 discarded as burn-in and parameter values sampled every 1,000 generations) were combined with LogCombiner v1.6.2 software (Drummond and Rambaut 2007). Posterior probability values provide an assessment of the degree of support of each node on the tree. The final tree was estimated using TreeAnnotator v1.6.2 software and visualized in the FigTree v1.3.1 program (Drummond and Rambaut 2007).

We tested the hypothesis that the squirrel monkeys fall into three possible morphological classificatory schemes: (1) Hershkovitz (1984)–Groves (2001); (2) Thorington (1985) and (3) Costello et al. (1993), and into three molecular classificatory schemes: (1) Cropp and Boinski (2000); (2) Lavergne et al. (2010) and (3) Chiou et al. (2011). These classifications were contrasted with our maximum likelihood tree. For this, we performed parametric bootstrapping and a posteriori significance test with the Swofford–Olsen–Waddell–Hillis test (SOWH; Huelsenbeck and Bull 1996; Swofford et al. 1996). The six hypotheses were employed as a model tree for parameter estimation and for generating 100 replicate data sets in the software Seq-Gen 1.2.5 (Rambaut and Grassly 1997) which presented a uniform base composition. Goldman et al. (2000) demonstrated that this procedure can increase power in rejecting the null hypothesis and is better than typical nonparametric tests for comparisons of a posteriori hypotheses. The differences between the log likelihood of these six hypotheses and the tree herein obtained, for both genes simultaneously taken, were compared with the distribution of the differences between each parametric replicate and the tree employed as representative of the six hypotheses. The same was performed with the Shimodaira and Hasegawa (1999) test (nonparametric SH test).

We used the Software Network 4.6.10 (Fluxus Technology Ltd.) to form a median joining network to estimate possible divergence times among the haplotypes (joined *mt COI* and *COII* sequences) in squirrel monkeys (Bandelt et al. 1999). The ρ statistic (Morral et al. 1994) was estimated and transformed into years. To determine the temporal splits, it is necessary to estimate the mutation rate at these *mt CO* genes. Ruvolo et al. (1991) determined a mutation rate of 0.85 % per million years per lineage for

Hominoidea at *mt COII*. This represents 1 mutation on average each 199,402 years. This mutation rate was practically identical to that determined by Ruiz-García and Pinedo-Castro (2010) in a *Lagothrix* study (1 mutation on average every 191,000 years). Similarly, for *Aotus*, Ashley and Vaughn (1995) and Ruiz-García et al. (2011) determined 1 mutation on average every 199,000 years in this same mitochondrial gene. For *mt COI*, an average mutation rate of 1 % per million years was employed (Matzen da Silva et al. 2011; Olson et al. 2009). This represents an average of 1 mutation each 152,000 years. Thus, we have used an average of one mutation each 171,600 years for both *mt COI* and *COII*. This corresponded approximately to 5.6×10^{-3} substitutions/site/million years, which is a typical mutation rate in many different mammal taxa (Thoisy et al. 2010).

Other procedures could be employed to estimate the temporal fragmentation of the *Saimiri* groups. Some authors, for example Chiou et al. (2011), have employed Bayesian estimations for this task. However, these estimations are extremely sensitive to the priors selected by the authors. To select well-established Bayesian priors, we need exact temporal fossil estimations, but we do not have exact estimations of this nature for the *Saimiri* genus (in the literature, the split between *Saimiri* and *Cebus* is very wide, 12–21 MYA). For this reason, we prefer to employ the ρ statistic, which is independent of exact paleontological estimations and it only depends on the exact estimation of the mutation rate of the molecular marker employed. We are confident that our mutation rate is accurate.

Population differentiation analyses

To determine possible differences among all the S*aimiri* pairs of groups found, we employed the ϕ_{ST} statistic (Weir and Hill 2002) with exact probabilities (Raymond and Rousset 1995). These population differentiation statistics were undertaken in the programs DNAsp 5.1 (Librado and Rozas 2009) and Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

Results

Characteristics and evolutionary models of the sequences studied

The nucleotide frequencies were $A = 31.8$ %, $T = 28.4$ %, $C = 26.3$ %, and $G = 13.5$ % for the 225 sequences and 2,237 bp analyzed at the *mt COII* gene. For the AIC procedure, the best evolutionary models were GTR (+G) and GTR (+G+I), which practically yielded the same results. The following evolutionary model was HKY (+G), but this model was clearly less adequate than the previous ones ($\Delta AIC = 10.66$). The maximum likelihood estimate of transition/transversion bias was 3.41 for the GTR (+G) model (−4680.397). The estimated value of the shape parameter for the discrete gamma distribution was 0.7783 for the GRT (+G) model (−4871.945).

Phylogenetic inferences

The four phylogenetic procedures we used indicated the existence basically of the same *Saimiri* groups for individual genes and for all the specimens studied (Supplementary data; two maximum likelihood trees for *mt COI* and *COII*, respectively) and for both genes concatenated and for haplotypes (Fig. 2; one maximum likelihood tree and one Bayesian tree for the haplotypes obtained). The differences among these procedures were the relationships among these different groups. All the animals classified "a priori" as *albigena* conformed to a monophyletic group as well as all the animals classified as *cassiquiarensis*. Both of these groups were sister taxa for all the different procedures applied. The animals classified a priori as *macrodon* by their phenotypes and geographical origins formed five different clusters. We named them *macrodon I*, *II*, *III*, *IV* and *V*. These *macrodon* groups were intermixed among other *Saimiri* taxa. Thus, *macrodon* sensu stricto is an artificial polyphyletic group. *Macrodon I* was clustered within the *albigena* group. Three of the four animals were from the Putumayo Department in Colombia and the other was from the Putumayo River (Flor de Agosto) in the northern Peruvian Amazon. *Macrodon II* and *III* are two groups strongly related to the clade *albigena and macrodon I—cassiquiarensis*. Both groups are established and living sympatrically in the Colombian and northern Peruvian Amazon. The *macrodon III* group had two animals with intermediate phenotype characteristics of *peruviensis* and *macrodon* but with mitochondrial haplotypes of *macrodon*. This could be a case of hybridization. *Macrodon IV* was composed of animals that originated in the Ecuadorian Amazon and in the central and southern Peruvian Amazon (Ucayali River). This group also had four animals with intermediate phenotype characteristics of *peruviensis* and *macrodon* but with *macrodon* mitochondrial haplotypes. *Macrodon IV* was more related in all the analyses with *ustus II* than with other *macrodon* groups. *Macrodon V* was exclusively composed of animals from the Colombian Amazon. The *ustus* group is also an artificial and paraphyletic one. Three different *ustus* groups were differentiated. *Ustus I* was composed of animals from the Purus and Amazon rivers. In many analyses, this *ustus* group is related with different *macrodon* groups, whereas *ustus II* and *ustus III* were composed of animals from the Madeira and Amazon rivers and were closely related to *macrodon*

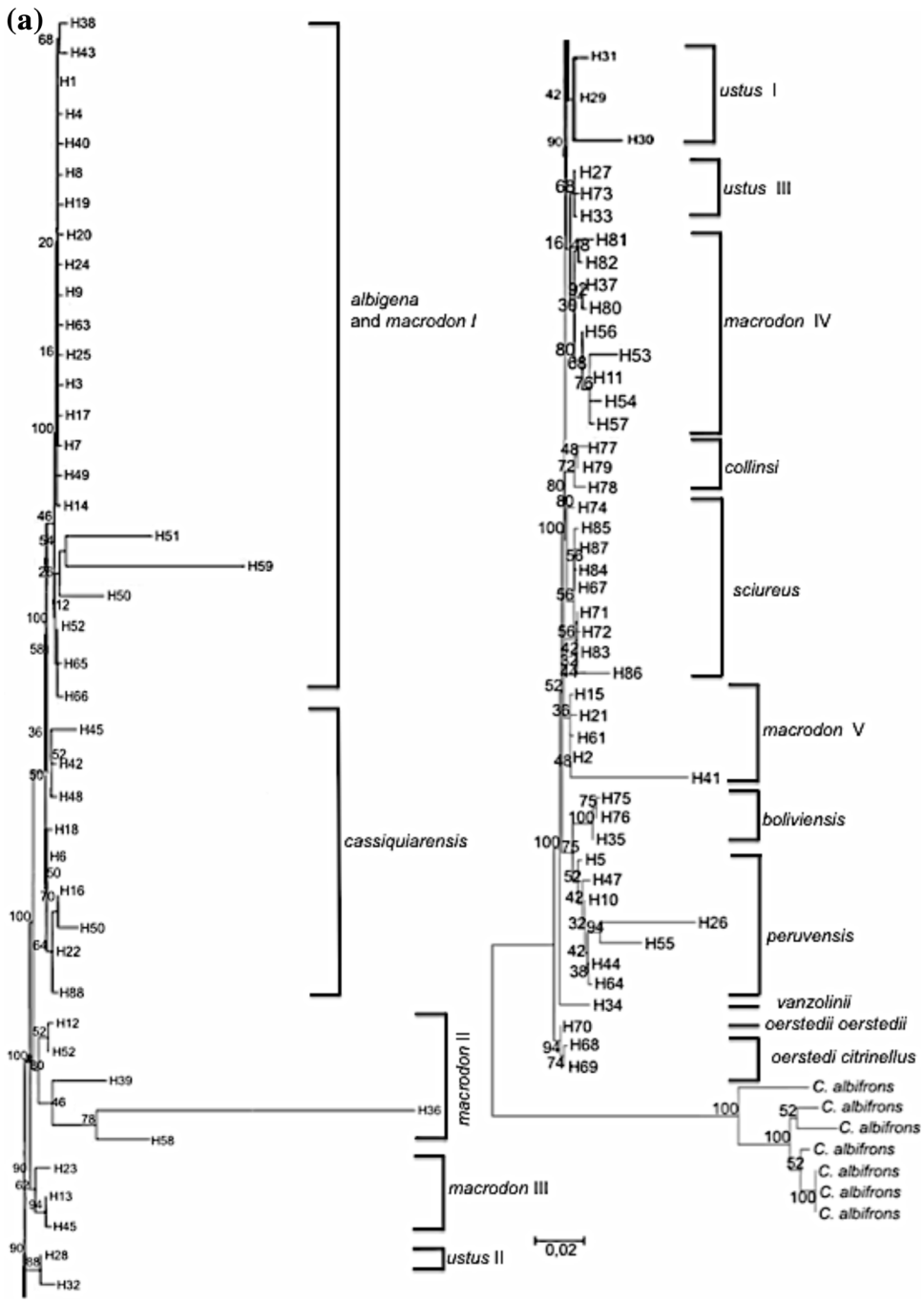
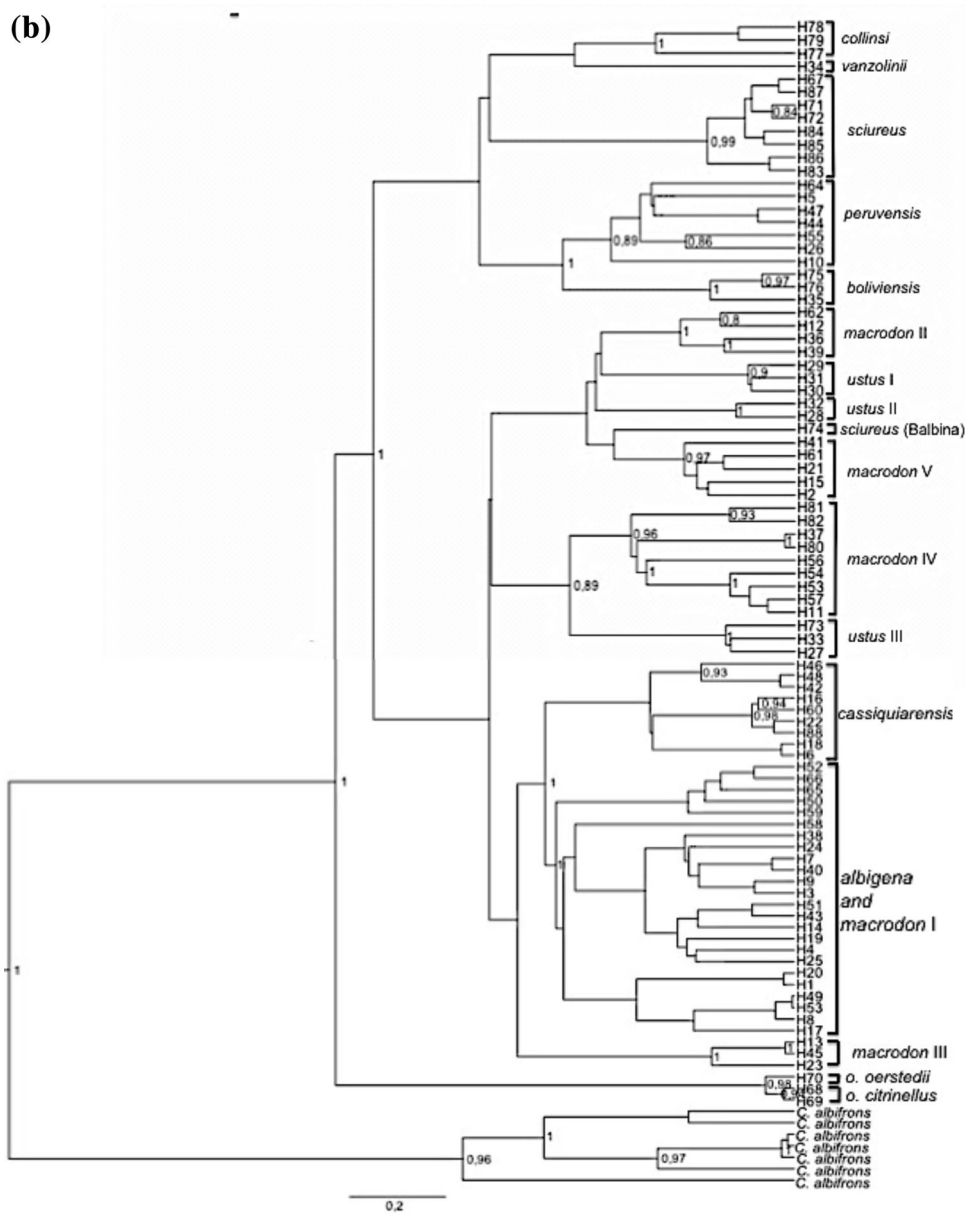


Fig. 2 **a** Maximum likelihood tree for the 88 *Saimiri* haplotypes simultaneously found at the *mt COI* and *COII* gene. Numbers in the nodes are bootstrap percentages. **b** Bayesian tree for the 88 *Saimiri* haplotypes simultaneously found at the *mt COI* and *COII* gene. Numbers in the nodes are posterior probabilities (only values higher than 0.5 are shown)

IV. Another group was composed of individuals belonging to *sciureus* from French Guyana. One individual within this phenotype was sampled in the northern Brazilian Amazon (Balbina) and is related with this group in some analyses, although other analyses assigned it more closely with other *macrodon* groups. Two animals within this *sciureus* group had *collinsi* phenotypes but with mitochondrial haplotypes typical of *sciureus*. This provides further evidence of possible hybridization between these two different *Saimiri* taxa. Another clade contained three *collinsi* individuals differentiated from *sciureus*. Another clear group consisted of *S. boliviensis.* Within this group, two clusters were detected—agreeing quite well with the traditional classifications of *boliviensis* and *peruviensis*. Within this last group, seven individuals presented some mixed phenotype characteristics between *macrodon* and *peruviensis* but with the *mtDNA* of *peruviensis*. Therefore, as stated previously, we detected possible hybridization between *peruviensis* and diverse *macrodon* groups. The unique *vanzolinii* individual we analyzed was of an independent lineage and not

Fig. 2 continued



integrated with any other *Saimiri* group. The Central American *Saimiri* individuals formed another differentiated cluster and within it, the two taxa proposed were detected (although we analyzed only three individuals): *oerstedii* and *citrinellus*.

As we previously stated, the main differences among these trees, obtained with two different mitochondrial genes and with different phylogenetic procedures, were the relationships among the different *Saimiri* groups and the relationships of certain individuals. These were the cases of the individual from Balbina, the *vanzolinii*exemplar, and the relationships of the *macrodon II* and the *ustus III* groups with other *Saimiri* taxa. However, all the different groups found were the same for both genes and for the different phylogenetic procedures employed as well as the major part of the relationships among these different groups.

The SOWH tests (and also the SH tests) indicated that there was no support for the morphological taxonomic schemes suggested by either Hershkovitz (1984)–Groves (2001) or by Thorington (1985). The maximum likelihood trees were significantly different at the 0.001 level (423,956 and 974,276 log likelihood units, respectively). However, the scheme suggested by Costello et al. (1993) did not significantly deviate from the tree we obtained (2,236 log likelihood units, $p < 0.40$). Thus, the Costello et al. (1993) scheme (two species, *S. oerstedii* and *S. sciureus*) is the most similar to the phylogenetic results that we obtained. All the previous molecular trees obtained by

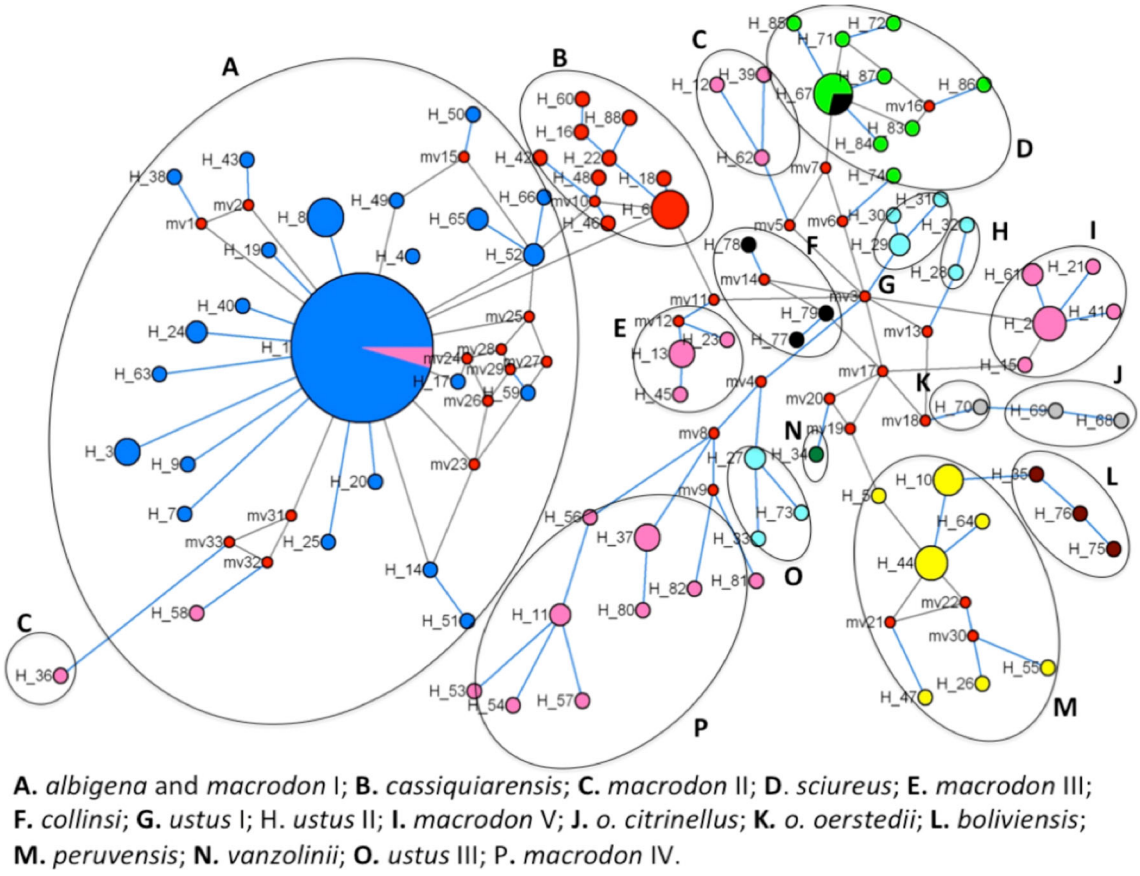


Fig. 3 Median joining network with the 88 mitochondrial haplotypes found in this study. *Grey, oerstedii* and *citrinellus*; *dark green, vanzolinii*; *dark blue, albigena*; *pink, macrodon I*, *II*, *III*, *IV*, *V*; *red, cassiquiarensis*; *yellow, peruviensis*; *brown, boliviensis*; *black, collinsi*; *light green, sciureus*; *light blue, ustus I*, *II*, *III*

other authors significantly diverged from our tree. However, the tree of Lavergne et al. (2010) was the most similar to our own (the other two hypotheses had log likelihood units of 87,354 and 465,476, respectively, $p < 0.001$). Therefore, the molecular tree of Lavergne et al. (2010) with the *mtCyt-b* gene, although significantly different from our tree, was the most similar. Thus, the two molecular studies with the highest number of individuals and taxa were also the most similar.

Divergence times

The MJN is shown in Fig. 3 and agrees quite well with the phylogenetic trees constructed. The star form of the MJN for *boliviensis*, *sciureus*, some *macrodon* groups but especially for *albigena* was in agreement with a population expansion. This analysis also showed that *macrodon* and *ustus* are composed of different genetic groups. We were able to determine the temporal split estimates within and among the different *Saimiri* groups using the ρ statistic. The highest temporal splits found were those implicating *boliviensis*, *vanzolinii* and *oerstedii*: *vanzolinii–albigena* around 1,560,000 YA (the same temporal splits for *vanzolinii–boliviensis* and *vanzolini–macrodon IV*), *boliviensis–albigena* around 1,462,500 (the same value for *boliviensis–macrodon IV* and *oersteddi–vanzolinii*). The lowest genetics temporal split was between *albigena* and *cassiquiarensis* at around 195,000 YA. The average temporal split within the different *macrodon* lineages was around 731,300 ± 182,300 YA and for the different *ustus* lineages was 390,000 ± 97,500 YA.

Population differentiation

Around 69 % of the F_{ST} pair tests with exact probabilities (83/120; Table 1) among the 16 *Saimiri* groups found (two *ustus* groups were unified) were significant, which indicates significant genetic heterogeneity among the majority of the *Saimiri* groups considered. For example, the population differentiation was significantly different between the *ustus* groups as well as among all the different *macrodon* groups, between the two *boliviensis* groups and

Table 1 ϕ_{ST} statistic pairs among 16 different *Saimiri* groups detected by the phylogenetic analyses

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	0.42*														
3	−0.13	0.40*													
4	0.71*	0.39*	0.18+												
5	0.73*	0.62*	0.84	0.23*											
6	0.75*	0.60*	0.63*	0.36*	0.53*										
7	0.73*	0.55*	0.57*	0.29*	0.46*	0.42*									
8	0.70*	0.54*	0.63*	0.18*	0.46*	0.37*	0.27*								
9	0.74*	0.66*	0.91*	0.16	0.72+	0.32*	0.37*	0.36*							
10	0.81*	0.68*	0.67*	0.41*	0.63*	0.60*	0.55*	0.55*	0.59*						
11	0.86*	0.82*	0.97*	0.23	0.87*	0.69*	0.64*	0.67*	0.89+	0.46*					
12	0.85	0.77	1.00	−0.22	0.83	0.61	0.52	0.55	0.88	0.59	0.92				
13	0.76*	0.68*	0.77*	0.36*	0.67*	0.54*	0.48*	0.46*	0.62*	0.61*	0.78*	0.74			
14	0.74*	0.60*	0.69*	0.16*	0.54*	0.43*	0.33*	0.28*	0.47*	0.51*	0.68*	0.55	0.28+		
15	0.81*	0.75*	0.97	0.06	0.81+	0.57+	0.48	0.500+	0.85	0.59+	0.92	0.93	0.68*	0.52+	
16	0.78	0.68	1.00	−0.42	0.74	0.43	0.29	0.26	0.80	0.49	0.90	1.00	0.58	0.27	0.33

1, *albigena*; 2, *cassiquiarensis*; 3, *macrodon I*; 4, *macrodon II*; 5, *macrodon III*; 6, *macrodon IV*; 7, *macrodon V*; 8, *ustus I*; 9, *ustus II*; 10, *peruviensis*; 11, *boliviensis*; 12, *vanzolinii*; 13, *sciureus*; 14, *collinsi*; 15, *citrinellus*; 16, *oerstedii*

+ $p < 0.05$; * $p < 0.001$

Table 2 Kimura 2P genetic distance pairs among nine morphological 1028 taxa of *Saimiri* recognized by Groves (2001) at the *mt COI–COII* genes

	alb	mac	cas	per	bol	oer	ust	sci	col
alb	–								
mac	0.0186	–							
cas	0.0066	0.0179	–						
per	0.0263	0.0277	0.0246	–					
bol	0.0256	0.0267	0.0240	0.0162	–				
oer	0.0185	0.0202	0.0170	0.0231	0.0217	–			
ust	0.0144	0.0160	0.0129	0.0219	0.0193	0.0127	–		
sci	0.0161	0.0175	0.0146	0.0202	0.0191	0.0122	0.0107	–	
col	0.0165	0.0181	0.0150	0.0207	0.0193	0.0130	0.0112	0.0084	–

alb albigena, *mac macrodon*, *cas cassiquiarensis*, *per peruviensis*, *bol boliviensis*, *oer oerstedii*, *ust ustus*, *sci sciureus*, *col collinsi*

between *sciureus* and *collinsi*. However, there was no significant genetic heterogeneity between *albigena* and *macrodon I* or between *macrodon II* and *ustus II* just as the phylogenetic analyses had indicated. *Vanzolinii* and *oerstedii* and the other groups did not show significant differences because both were only grouped by a unique sequence and their power of discrimination was minimal. However, the genetic distances found were relatively small. Table 2 shows the Kimura 2P genetic distances (Kimura 1980) among all "a priori" *Saimiri* taxa. The highest genetic distances were between the *boliviensis* groups and all the other *Saimiri* taxa ($D = 0.026$–0.028). The two lowest genetic distances were between *sciureus* and *collinsi* ($D = 0.0084$) and between *albigena* and *cassiquiarensis* ($D = 0.0066$).

Discussion

The most relevant contribution of the present work to resolve *Saimiri* molecular systematics was the use of the largest sample used to date, with 218 "in situ" specimens collected across *Saimiri*'s distribution range over the last 14 years for 2,237 bp of two mitochondrial genes, as well as that, for first time, all the morphologically recognized taxa of *Saimiri* were included in a molecular phylogenetic study. Our work provides new results of *Saimiri* phylogeny not previously reported in other molecular studies. However, there is a need to complete other additional molecular genetics markers that focus on *Saimiri* systematics and thus help us to better understand the evolutionary history of this genus.

However, some care should be taken when using only mitochondrial genes for resolving taxonomic problems because gene trees do not necessarily correspond well with species trees. Species can diverge simultaneously with a pair of mitochondrial haplotypes or they can diverge after a pair of haplotypes diverged. However, it is possible that some time after a population divides, that a new haplotype may appear in the gene tree. A migrant could carry the new haplotype to the other population and, then, the new haplotype is lost in one population and the ancestral haplotype is lost in the other. Therefore, if we use the gene tree to estimate genetic heterogeneity and the divergence time for the species tree, the species will appear to have diverged more recently than they really did (Freeman and Herron 1998). Furthermore, mitochondrial genes only show the evolutionary history of the female lineages, and this could miss hybridization events between close species or groups when males are the unique gene flow vectors ('mitochondrial capture'; Burrell et al. 2009).

For this reason, in order to have a more definitive understanding of the total *Saimiri* phylogeny and its evolutionary history, it would be useful to apply additional molecular markers, such as nuclear DNA and MHC genes as well as introns of chromosomes *X* and *Y*.

Main phylogenetic findings in *Saimiri*

Our study showed from a phylogenetic perspective the following noteworthy aspects:

1. Similar to findings by Lavergne et al. (2010) using *mt Cyt-b*, both *albigena* and *cassiquiarensis*, were monophyletic, and closely related. The geographical separation of these two *Saimiri* groups is a result of an extension of the Colombian Eastern Llanos and the upper Guaviare River. Although they have different phenotypes (*cassiquiarensis* has a fairly weak contrasting light nucal collar, tawniness in its crown and a different skull that is correlated with absolute size; Thorington 1985), they are closely related from a molecular point of view. In fact they were the two traditionally recognized *Saimiri* taxa more related from a molecular genetics point of view. Probably, *albigena* is the most recent *Saimiri* group, which is correlated with the lowest values of gene diversity ($\pi = 0.0024$) of all the lineages we analyzed, meanwhile the second more recent *Saimiri* lineage is *cassiquiarensis* ($\pi = 0.0048$).

2. The *boliviensis* group has multiple characteristics, which differentiate them from the other *Saimiri* groups. This group has different acoustic structure and vocalization perception compared to other *Saimiri* (Boinski and Newman 1988). Additionally, there are behavioral differences between *boliviensis* and other *Saimiri* taxa — adult males segregating outside mating season, females dominating males, and females forming cohesive groups based on matrilineages that defend territory and food. *Boliviensis* females also show complete philopatry behavior whereas *S. sciureus* females migrate (Hershkovitz 1984; Boinski and Cropp 1999). Although the differences among the haplotypes of *boliviensis* and *peruviensis* were small, they formed two well-defined clades, which is consistent with that determined by Boinski and Cropp (1999), Cropp and Boinski (2000) and Lavergne et al. (2010). This differentiation is also consistent with that obtained from karyotypic studies, where *boliviensis* has six acrocentric autosomal pairs whereas *peruviensis* has five acrocentric autosomal pairs (Groves 2001). However, although the *boliviensis* group has its own differentiated characteristics, there is extensive hybridization among *peruviensis* and different *macrodon* lineages, as we will comment below.

3. *Oerstedii* was the most differentiated *Saimiri* taxon with the two traditional subspecies, *oerstedii* and *citrinellus*, although only three Central American individuals were analyzed. Similar to the findings of Costello et al. (1993), (Boinski and Cropp 1999), Cropp and Boinski (2000), Lavergne et al. (2010) and Chiou et al. (2011), this is a well-conformed group.

4. Although we only sequenced one exemplar of *vanzolinii*, this is the first molecular result for this taxon. Apparently, *vanzolinii* formed an independent lineage from other *Saimiri* lineages, although more molecular results are pending to adequately confirm this. However, *vanzolinii* has some chromosome peculiarities, such as the presence of heterochromatin blocks in the long arm of chromosomes 13, 15, 17 and 19 and the absence of an interstitial C-band in the short arm of chromosome 6, which differentiates it from other *Saimiri* taxa (Yonenaga-Yassuda and Chu 1985).

5. *S. sciureus* is considered by some authors to be the ancestor of the other *Saimiri* taxa. Moore et al. (1990), following the reasoning of Dutrillaux and Couturier (1981) and Dutrillaux (1988) for *Cebus*, deduced that *sciureus* with seven pairs of acrocentric chromosomes (15 and 16 chromosomes were acrocentric) was the original karyotype of *Saimiri* because the direction of chromosomal change was acrocentric to submetacentric (Ma et al. 1974). Similarly, Hershkovitz (1984) determined that the primitive coat color is that observed in *sciureus*. However, many chromosome evolutionary studies have been discarded when molecular phylogenetic studies have been carried out (see, for instance, the case of *Aotus*, Ruiz-García et al. 2011) and the same has happened with color pattern studies. Dobzhansky (1971) demonstrated that the original populations have the highest diversity levels. Our results, as well as those of Lavergne et al. (2010) and Silva et al. (1993), did not show *sciureus* as one the forms of *Saimiri* with the highest gene diversity levels. Thus, we negate *sciureus* as a *Saimiri* taxon playing an important role in the

origin of other *Saimiri* lineages. One *sciureus* individual from the northern Brazilian Amazon was not clearly associated with the French Guyana group. In some analyses, this specimen was even more related to some *macrodon* and *ustus* lineages. This could provide proof of hybridization among *sciureus* and some lineages included within the classical *macrodon* and *ustus* groups or that, inside *sciureus*, there are significantly different molecular lineages as observed in *macrodon* and *ustus*.

6. The taxon *collinsi* was not recognized in the last classifications of *Saimiri*. For instance, Groves (2001) assimilated *collinsi* within *sciureus.* Our analysis determined that *collinsi* is in fact related to *sciureus* but it is differentiable from this last *Saimiri* group. However, two *collinsi* individuals presented the *mtDNA* of *sciureus* which is an indication of hybridization or historical introgression of *sciureus* and *collinsi* and/or a very recent split between these two *Saimiri* groups.

7. *Ustus* is a polyphyletic group constituted by at least three different molecular lineages. Some exemplars sampled between Coarí, at the Amazon River, and diverse localities at the Purus River formed one lineage. Two other lineages overlapped and were placed near the confluence of the Negro and Amazon Rivers, at the southern bank of the Amazon River and throughout the Madeira River. Silva et al. (1993), with protein systems, determined remarkable differences among two populations of *S. ustus* inhabiting the two banks of the Jamari River for the *ADA* and the *CA2* markers. This agrees quite well with the significant degree of heterogeneity that we observed between different *ustus* lineages.

8. Our study clearly demonstrated the existence of five different groups within the artificial group named *macrodon*. This is the first time that a molecular study has shown this high genetic heterogeneity within this *Saimiri* taxon. Boinski and Cropp (1999), and Cropp and Boinski (2000) did not analyze any *macrodon* samples, whereas Chiou et al. (2011) only analyzed one Ecuadorian *macrodon* sample. Thus, these works did not make any inference as to the real relationships of this *Saimiri* taxon to the other ones. In the current work (36 *macrodon* individuals analyzed), we can affirm that this is the *Saimiri* group with the highest within gene diversity, because it is composed of several polyphyletic molecular lineages. The most divergent group, for many trees, was *macrodon V*, which included animals sampled in three Colombian Amazon Departments (Amazonas, Putumayo and Caquetá), followed by *macrodon IV,* which was constituted by animals sampled in the Ecuadorian Amazon, northern (Amazon River) and central (Ucayali River) Peruvian Amazon and in the Amazon Department in Colombia. The most recently diverging *macrodon* group, for all the analyses, was *macrodon I*, constituted by animals from the Putumayo River in Colombia and Peru. Different divergent *macrodon* mitochondrial haplotype lineages live in sympatry in diverse Amazonian areas, as was previously found for another Neotropical monkey, *Cebus albifrons* (Ruiz-García et al. 2010).

Possible hybridization or historical introgression within *Saimiri*

We observed possible hybridization, or historical introgression, between *albigena* and *macrodon I* and between *macrodon V* and *sciureus* (the individual from the northern Brazilian Amazon). In the first case, two explanations can be generated. *Macrodon I* could have generated the western *albigena* population (Huila and Cauca Departments in the upper Magdalena valley: historical introgression) and this population in turn could have generated the eastern Colombian Llanos *albigena* population. The other possibility could be hybridization between the western *albigena* population and the *macrodon I* group, which in fact are geographical neighbors. If this last explanation was right, the hybridization was between *albigena* females and *macrodon I* males. This divergence and/or hybridization between these populations occurred recently because the haplotypes are the same or very similar. Other cases possibly showed female migration and hybridization among the different *Saimiri* groups considered. These were the cases of *peruviensis* and *macrodon III*, of *peruviensis* and *macrodon IV*, from *sciureus* into *collinsi* and from one *macrodon* lineage, at least, into *peruviensis*. Additionally, different groups of *ustus* intermixed with different groups of *macrodon*, which indicates that different *macrodon* and *ustus* lineages probably hybridized in different areas of the Amazon basin. While hybridization is one possible explanation, other possible explanations include incomplete lineage sorting, or historic introgression (to be differentiated from recent hybridization). However, we believe that many of the cases that we reported are caused by recent hybridization. Firstly, all the animals in which we detected possible hybridization were in areas of confluence of diverse *Saimiri* taxa and no case was detected between *Saimiri* taxa where they were not geographical neighbors. Secondly, the animals we detected as hybrids showed in many cases a mixture of morphological characters of the different *Saimiri* taxa implicated (photos of these exemplars can be requested, and morphological descriptions are shown in Appendix 1). Thirdly, some cases of hybridization among *Saimiri* taxa have been reported in the literature. Hershkovitz (1984) mentioned the possibility of sympatry between *ustus* and *sciureus* in the lower Madeira river basin, which Ruiz-García (unpublished observations) recently confirmed (January 2012). In fact, some troops contained animals with both phenotypes (Ruiz-García,

unpublished observations). Thorington (1985) recorded four localities where *ustus* and *sciureus* hybridize on the east bank of the Tapajos River. Costello et al. (1993) determined that *ustus* and *sciureus* produce hybrids between the Madeira and Tapajos rivers. Additionally, the same authors stated that *ustus* and *macrodon* hybridize east and west of Tefè Lake in the Brazilian Amazon. However, the major part of *Saimiri* hybridization cases reported in our study implicated *peruviensis* and *macrodon.* Our results showed that, at the level of the Ucayali River, both *Saimiri* taxa were found and there was a hybrid zone. This clearly supports the vision of Hershkovitz (1984) and Groves (2001). Additionally, one of the authors (Ruiz-García) confirmed Hershkovitz's (1984) claims. In the Tapiche River, as well as in other tributaries of the Ucayali River, both *Saimiri* phenotypes co-exist sympatrically in different and within the same troops. Other authors also found evidence of hybridization among *peruviensis* and *macrodon* (Silva et al. 1992; Jones et al. 1973). Silva et al. (1992) studied 49 exemplars from the overlapped area in Peru where *peruviensis* and *macrodon* live. By analyzing biochemical markers, these authors determined that 22 out of 49 exemplars (45 %) showed clear indications of admixture. The rivers have been suggested to act as barriers to the distribution of several genera and species of Neotropical primates (Ferrari 1993, 2004). Nonetheless, *Saimiri* has a strong potential for colonization and it is possible that in many cases, rivers are not sufficient barriers to prevent hybridization among different *Saimiri* groups. We strongly agree with Thorington (1985) that squirrel monkeys would be insensitive to the potential effect of river barriers due to their preferred distribution along watercourses. However, the use of nuclear markers could be useful for distinguishing real cases of recent hybridization against cases of incomplete lineage sorting or historic introgression.

Taxonomic implications

We are in agreement with the biological species concept (BSC) to define different species rather than other species definitions such as the phylogenetic or genetics species concepts (PSC and GSC), when reproductive data among taxa is available such as it is here for many *Saimiri* groups.

Taking into account the possible extensive hybridization detected among many South American *Saimiri* taxa, there is no clear consensus among the different relationships of the diverse *Saimiri* groups detected in the phylogenetic trees. One interesting fact is that the genetic distances we estimated among the different *Saimiri* groups we detected were relatively small or very small. Our genetic distances were obtained for both *mt COI* and *COII* and they were similar and they could be compared with other results obtained for primates and mammals, in general. Kartavtsev (2011) analyzed sequences of *mt COI* from 20,731 vertebrate and invertebrate animal species. He estimated the average distance data for five different groups. He obtained 0.89 ± 0.16 % for populations within species, 3.78 ± 1.18 % for subspecies or semi-species, 11.06 ± 0.53 % for species within a genus; 16.60 ± 0.69 % for species from different genera within a family and 20.57 ± 0.40 % for species from separate families within an order. Interestingly, Bradley and Baker (2001), focused their work on *mtCyt-b*, a gene with a similar behavior to the two mitochondrial genes we studied. They claimed values <2 % would equal intraspecific variation, values between 2 and 11 % would merit additional study, and values >11 % would be indicative of specific recognition. The highest genetic distances we obtained were between *macrodon* and both *boliviensis* groups (2.67–2.77 %), between *albigena* and these two *boliviensis* taxa (2.56–2.63 %) and between *oerstedii* and *peruviensis* (2.3 %). The remaining values were lower than 1 % or between 1 and 2 %. Thus, these genetic distances are lower than those of typical well-defined species. The same was found when we only compared for *mt COII*. Ascunce et al. (2003) estimated an average genetic distance of 0.032–0.033 between *Ateles* species, of 0.038–0.06 between *Alouatta* species or even values considerably higher between *Aotus* species (0.081–0.167). Our highest genetic distances were around 0.027, as we previously commented. Therefore, our estimates ranged within the typical subspecies values estimated for other Primates and other mammals and vertebrates at both mitochondrial genes. Although the highest genetic distances we determined were between *peruviensis* and other *Saimiri* taxa, such as *macrodon*, we precisely detected the major part of hybridization events between these two most genetically divergent taxa. This could be a proof, but an important one, that there is only one or very few *Saimiri* species.

Taking these results (small or very small genetic distances and extensive hybridization) combined with the consideration that reproductive isolation is a critical criterion in the BSC (Mayr 1942), make us question whether many of the different *Saimiri* lineages determined are really different species, as was proposed by Hershkovitz (1984)–Groves (2001). We have found the same for other South American primates, such as *Cebus albifrons* and *Cebus apella* (Ruiz-García et al. 2010, 2012a. The general consensus among scientists regarding humans is that there is only one species (*Homo sapiens*) with no living subspecies, because interbreeding has been clearly demonstrated, although very noteworthy morphological differences exists among different human groups, and there is no attempt to apply the PSC indiscriminately, as has been done to many Primates taxa in the last few years. If

this is the view for our own primate species, then we must disagree with other scientists that apply different species rules for other primate species. The BSC should not be ignored just because in many cases it is not easily translated into an operational definition. However, in the case of *Saimiri,* we have abundant data of reproductive interchange among different phenotype groups in the wild and so this definition is operational. In consideration of this, our results agree more strongly with the conclusion reached by Costello et al. (1993), that two species can be differentiated: the Central American squirrel monkey (*S. oerstedii*) and the South American squirrel monkey (*S. sciureus*). These are geographically isolated and no hybrids between *S. oerstedii* and other *Saimiri* groups have been located in the wild. Costello et al. (1993) determined two different molar patterns: one in the Central American squirrel monkey and another in the South American squirrel monkey. For this last pattern, a clinal distribution was observed from *sciureus* to *boliviensis*. The molar patterns for *sciureus* and *ustus* from the same area were practically undifferentiable. Biochemical data also showed *oerstedii* as the most differentiated group. Thus, the Gothic-arch and the Roman-arch characters do not have any phylogenetic value if our conclusion is correct.

In agreement with this, we propose the following *Saimiri* classification: *S. oerstedii*, with two subspecies, *S. o. oerstedii* and *S. o. citrinellus*; *S. vanzolinii* (if no hybridization can be demonstrated with other *Saimiri* groups) and *S. sciureus,* with two subspecies, *S. s. boliviensis* [with two lineages: 1 (*boliviensis*) and 2 (*peruviensis*)] and *S. s. sciureus* [with 12 lineages: 1 (*sciureus*), 2 (*cassiquiarensis*), 3 (*ustus I* = A), 4 (*ustus II* = B), 5 (*ustus III* = C), 6 (*macrodon I* = D), 7 (*macrodon II* = E), 8 (*macrodon III* = F), 9 (*macrodon IV* = G), 10 (*macrodon V* = H), 11 (*collinsi*) and 12 (*albigena*); the use of letters could be justified because *ustus* and *macrodon* there are not really real groups]. An alternative classification for the last species (*S. sciureus*) could include three subspecies, *S. s. boliviensis* (with the two aforementioned lineages), *S. s. sciureus* [with two lineages: 1 (*sciureus*) and 2 (*collinsi*)] and *S. s. cassiquiarensis* [with 10 lineages: 1 (*cassiquiarensis*), 2 (A), 3 (B), 4 (C), 5 (D), 6 (E), 7 (F), 8 (G), 9 (H), and 10 (*albigena*)]. Even a classification with one unique *Saimiri* species, with three to five subspecies (*oerstedii*, *vanzolinii*, *boliviensis*, *sciureus* and *cassiquiarensis*) could agree with the molecular results here obtained (for instance, with the small genetic distances estimated).

Temporal splits within *Saimiri*

The estimations of temporal splits of the different *Saimiri* groups could be explained by diverse geological and climatological reasons. The oldest temporal splits we detected within the *Saimiri* genera were around 1.4–1.6 MYA, implicating *oerstedii*, *vanzolinii* and *boliviensis*. This estimation is practically identical to that reported by Chiou et al. (2011) (1.5 MYA), and considerably lower than that obtained by Cropp and Boinski (2000), 4.05–6.42 MYA for *mt DNA*, or Lavergne et al. (2010), at the *mt Cyt-b* gene, with an average of 4.3 MYA (range 2.4–6.6 MYA).

The genetic diversification of *Saimiri* is considerably more recent than in other Neotropical Primates. *Ateles* began about 3.3–3.6 MYA (Collins and Dubach 2000), *Alouatta* diversified in northern South America around 2.9–3.3 MYA (Cortes-Ortiz et al. 2003), whereas the *Lagothrix*'s diversification began around 2.4–2.5 MYA (Ruiz-García and Pinedo-Castro 2010; Ruiz-García et al. 2014).

Between 1.5–0.7 MYA (the Pre-Pastonian glacial period), there was a period of intense diversification and fragmentation of haplotypes in *Saimiri* because the climate was considerably colder and drier. In many Neotropical mammals, this is a period of intense mitochondrial diversification and fragmentation, as revealed for the Pampas cat (Cossíos et al. 2009), for the jaguarundi and for foxes of the *Pseudoalopex* genus (Ruiz-García and Pinedo-Castro 2013; Ruiz-García et al. 2013).

Another very cold and dry epoch existed 0.5–0.3 MYA called the Mindel, Kansas, Elster or Kamasiense I glacial period (depending on the area of the planet analyzed). This was followed by an interglacial period (0.3–0.25 MYA; Hoxniense or Yarmouthian interglacial period with some dry peaks at 0.30 and 0.26 MYA; Van der Hammen et al. 1991; Van der Hammen 1992). During these periods, the *Saimiri* haplotype fragmentation and diversification was also intense, for instance the temporal divergence of the *ustus* lineages and others. The Pleistocene forest refugia invoked by Haffer (1969, 1982) could be very important for understanding the evolution of the squirrel monkeys.

For a more complete picture of the phylogeny of the *Saimiri* genus, it is important to obtain representative samples of *oerstedii*, *vanzolinii* and individuals sampled in and between the Jurua and Purus rivers, in and between the Tapajos and the Iriri rivers, in and between the Xingu and Tocantins rivers and throughout the Negro River.

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