Pleistocene diversification of living squirrel monkeys (*Saimiri* spp.) inferred from complete mitochondrial genome sequences

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Abstract

In order to enhance our understanding of the evolutionary history of squirrel monkeys (*Saimiri* spp.), we newly sequenced and analyzed data from seven complete mitochondrial genomes representing six squirrel monkey taxa. While previous studies have lent insights into the taxonomy and phylogeny of the genus, phylogenetic relationships and divergence date estimates among major squirrel monkey clades remain unclear. Using maximum likelihood and Bayesian procedures, we inferred a highly resolved phylogenetic tree with strong support for a sister relationship between *Saimiri boliviensis* and all other *Saimiri*, for monophyly of *Saimiri oerstedii* and *Saimiri sciureus sciureus*, and for *Saimiri sciureus macrodon* as the sister lineage to the *S. oerstedii/S. s. sciureus* clade. We inferred that crown lineages for extant squirrel monkeys diverged around 1.5 million years ago (MYA) in the Pleistocene Epoch, with other major clades diverging between 0.9 and 1.1 MYA. Our results suggest a relatively recent timeline of squirrel monkey evolution and challenge previous conceptions about the diversification of the genus and its expansion into Central America.

1 Introduction

Squirrel monkeys (*Saimiri* spp.) are small, forest-dwelling primates of the New World tropics. In South America, they are a widespread group of New World monkeys (parvorder Platyrrhini), with a wide distribution encompassing most of the Amazon Basin and the Guianas. Central American squirrel monkeys (*Saimiri oerstedii*), by contrast, occupy a narrow and disjunct area along the western edges of Costa Rica and Panama (Fig. 1). All squirrel monkeys live in large multimale-multifemale social groups, with group sizes ranging from 20 to 50 animals. They are versatile in the wild, inhabiting a wide range of forest environments including some disturbed habitats (Boinski, 1987). Squirrel monkeys are flexible omnivores, with diets including insects, fruits, flowers, eggs, and even small vertebrates (Janson and Boinski, 1992). In captivity, their small size and ease of handling have contributed to their status as the most commonly studied platyrrhine in American biomedical research (Williams et al., 2010). Despite this, their intrageneric taxonomy has been contentious, undergoing frequent revision in recent decades (e.g., Hershkovitz, 1984; Thorington, 1985; VandeBerg et al., 1990; Costello et al., 1993; Silva et al., 1993; Boinski and Cropp, 1999; Lavergne et al., 2010).

Despite forming distinct geographic variants based on a wide array of morphological, molecular, and behavioral traits (Boinski and Cropp, 1999), the distribution of physical variation along continuous gradients (e.g., Thorington, 1985) and the apparent ability of distinct forms to hybridize in both captivity (Fogle, 1990; Schreiber et al., 1998; Lavergne et al., 2003) and the wild (Thorington, 1985; Silva et al., 1992, 1993; Costello et al., 1993) have complicated the study of squirrel monkey systematics. Different studies have recognized from one to seven species and up to seventeen subspecies of *Saimiri* (Costello et al., 1993). Of these, Hershkovitz's (1984) classification has received the most attention in the literature and has largely been upheld by molecular and genetic evidence (VandeBerg et al., 1990; Boinski and Cropp, 1999; Lavergne et al., 2010; but see Silva et al., 1993). Under Hershkovitz's taxonomy, nine taxa including four species (*Saimiri sciureus*, *Saimiri boliviensis*, *Saimiri ustus*, and *Saimiri oerstedii*) and eight subspecies are recognized (Fig. 1).

Genetic studies have helped elucidate phylogenetic relationships among squirrel monkey populations (Boinski and Cropp, 1999; Cropp and Boinski, 2000; Lavergne et al., 2010), supporting a sister relationship between the two subspecies of *S. boliviensis* (*Saimiri boliviensis boliviensis* and *Saimiri boliviensis peruviensis*) and another between Guianan *S. sciureus* (*Saimiri sciureus sciureus*) and the two subspecies of *S. oerstedii* (*Saimiri oerstedii oerstedii* and *Saimiri oerstedii citrinellus*). In a recent analysis of mitochondrial cytochrome *b* sequences, Lavergne et al. (2010) identified a third clade comprising the three western Amazonian subspecies of *S. sciureus* (*Saimiri sciureus macrodon*, *Saimiri sciureus cassiquiarensis*, and *Saimiri sciureus albigena*) and a fourth clade comprising *S. ustus* and *Saimiri sciureus collinsi*, a subspecies of *S. sciureus* not included in Hershkovitz's (1984) classification. In their study, however, relationships among the four major clades could not be conclusively resolved.

As with all platyrrhines, the squirrel monkey paleontological record is entirely absent from the Late Miocene to the terminal Pleistocene. Among South American fossil fauna, the Middle Miocene genus *Neosaimiri* from La Venta, Colombia, dated to 12.1-12.5 MYA (Hartwig and Meldrum, 2002), has been most closely and consistently linked with extant
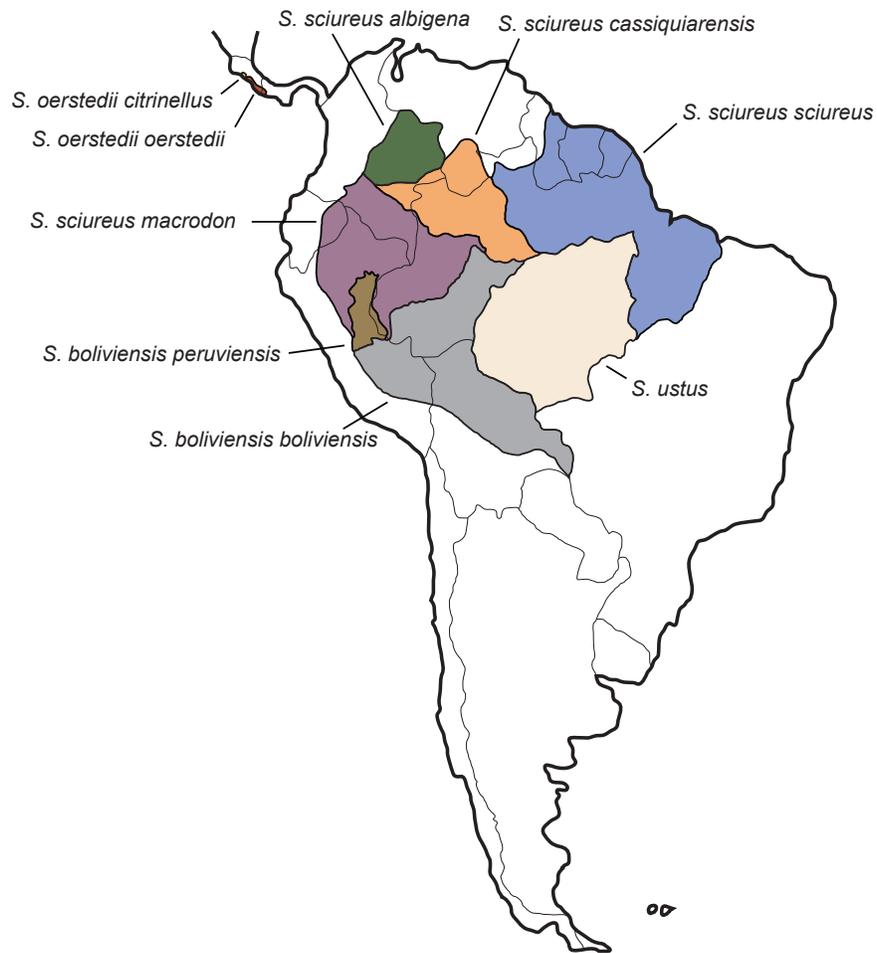
Figure 1: Geographic distribution of four species and eight subspecies of *Saimiri* based on the taxonomy of Hershkovitz (1984).

Saimiri based on similarities in dental (Stirton, 1951; Hershkovitz, 1970; Rosenberger et al., 1991; Takai, 1994) and postcranial morphology (Nakatsukasa et al., 1997). The Early Miocene fossil *Dolichocebus* from Gaiman, Argentina, dated to over 20.5 MYA (Fleagle and Tejedor, 2002), has also been linked to *Saimiri* based on similarities in cranial morphology (Rosenberger, 1979). With a time depth of over 20 million years, the three-genus evolutionary sequence of *Dolichocebus-Neosaimiri-Saimiri* has been suggested as one of the longest anagenetic lineages known from the primate fossil record (Delson and Rosenberger, 1984), although this idea has been met with controversy (Kay et al., 2008; Hodgson et al., 2009; but see Rosenberger, 2010).

In the absence of fossil data, molecular phylogenies offer the opportunity to evaluate the tempo and mode of speciation in extant taxa (Moritz et al., 2000). A number of nonexclusive hypotheses have been proposed to explain the origins of species richness in the Neotropics. Of these, the refuge hypothesis (Haffer, 1969) has been most widely discussed. Under the refuge model, past climatic changes caused rainforests to contract to isolated refuge zones separated by dry forest and savanna environments, creating favorable conditions for speciation. Attention on the refugia model has largely focused on the most recent glacial cycles in the Pleistocene. Molecular studies, however, suggest that most speciation events in tropical rainforest birds, mammals, lizards, frogs, and salamanders predate the Pleistocene (Moritz et al., 2000). For pre-Quaternary speciation events in both Neotropical fauna and flora, attention has focused on the role of geological events such as the Andean orogeny and the completion of the Isthmus of Panama (Colinvaux and De Oliveira, 2001; Nores, 2004; Rull, 2006, 2008; Hoorn et al., 2010).

The Andean orogeny played a critical role in determining which South American taxa migrated into Central America following the completion of the Isthmus of Panama. Based on present distributions, precursors of Central American howler monkeys, spider monkeys, owl monkeys, capuchin monkeys, and tamarins may have been isolated from ancestral South American populations by the Cordillera Oriental in Colombia and Venezuela around 8-10 MYA; therefore, these were the taxa available to migrate following the completion of the Isthmus (Ford, 2006). Genetic studies of spider monkeys (Collins and Dubach, 2000) and howler monkeys (Cortés-Ortiz et al., 2003) are congruent with these observations and suggest that both were relatively early participants in migrations into Central America. A recent cytochrome *b* analysis of capuchin monkey phylogeography, in contrast, puts the capuchin radiation into Central America much later, at ∼1.9 MYA (range 1-3 MYA) (Lynch Alfaro et al., accepted for pubication). Notably, squirrel monkeys are absent in northwestern Colombia yet present along the western edges of Costa Rica and Panama. Hershkovitz (1969, 1984) suggested that pre-Columbian human traders might have been responsible for introducing squirrel monkeys (*S. oerstedii*) to Central America, accounting for their peculiar distribution and habituation to human presence. Based on nuclear and mitochondrial divergences predating the arrival of humans in the Americas (Cropp and Boinski, 2000), however, Ford (2006) posited that ancestors of *S. oerstedii* were indeed present in northwestern Colombia around the late Miocene or Pliocene and participated in the earliest migrations into Central America around 3.0-3.5 MYA.

In this study, we reevaluate the phylogeny and historical biogeography of squirrel monkeys using newly determined complete mitochondrial genome sequences. Because appropriate choice of calibration points is one of the most critical factors for estimating divergence

Table 1: Subjects sampled in this study

Taxon and identifier	ID	Sample type	Locality	Source[a]
S. sciureus macrodon	391	Tissue	Tiputini Biodiversity Station, Ecuador	AD
S. sciureus sciureus	SG	Blood	Guyana (captive population)	UTMDACC
S. boliviensis peruviensis	SP	Blood	Peru (captive population)	UTMDACC
S. boliviensis boliviensis	SB	Blood	Bolivia (captive population)	UTMDACC
S. oerstedii oerstedii	So36	Fecal	Golfito, Costa Rica	GGE
S. oerstedii citrinellus 1	P2	Fecal	Manuel Antonio, Costa Rica	MB
S. oerstedii citrinellus 2	E19	Fecal	Esterillos, Costa Rica	MB

[a] AD, Anthony Di Fiore; UTMDACC, University of Texas MD Anderson Cancer Center; GGE, Gustavo Gutiérrez-Espeleta; MB, Mary Blair.

dates (e.g., Raaum et al., 2005; Steiper and Young, 2006, 2008; Benton and Donoghue, 2007; Ho and Phillips, 2009), we first review the primate fossil record to obtain a set of unambiguously placed fossil calibration constraints. Then, using complete sequence data from all 12 mitochondrial heavy strand protein-coding genes, we infer divergence dates in an attempt to time the diversification of the squirrel monkey genus.

2 Materials and methods

2.1 Taxon sampling

Samples were selected for complete mitochondrial DNA (mtDNA) sequencing with the purpose of completing a phylogenetically balanced dataset with representatives from all putative squirrel monkey species and subspecies, following Hershkovitz's (1984) treatment of taxonomic names for *Saimiri*. Altogether, seven squirrel monkey blood, tissue, and fecal samples were obtained, representing three of four species and six out of eight subspecies outlined by Hershkovitz (1984) (Table 1).

2.2 DNA purification

High molecular weight cellular DNAs were extracted and isolated using protocols specific to each sample type. For blood and tissue samples, the QIAamp DNA Mini Kit (Qiagen, Inc.) was used. Blood extractions followed the protocol "DNA Purification from Blood or Body Fluids (Spin Protocol)" (11/2007 version) while tissue extractions followed the protocol "DNA Purification from Tissues" (11/2007 version). Both protocols were modified as follows: (1) Buffer AE was heated to 70 °C prior to elution and (2) the QIAamp Mini spin column was incubated at 22 °C for 20-25 min. after adding Buffer AE. For fecal samples, the QIAamp DNA Stool Mini Kit (Qiagen, Inc.) was used, following the protocol "Isolation of DNA from Stool for Human DNA Analysis" (07/2007 version) with the following modifications: (1) fecal samples were incubated at 22 °C for 30-60 min. after adding Buffer ASL, (2) one-half of the InhibitEX Tablet was used instead of a full tablet, (3) samples were centrifuged for 6 min. after adding the InhibitEX Tablet, (4) samples were incubated at 70 °C for 30 min. after adding Buffer AL, (5) Buffer AE was heated to 70 °C prior to elution, (6) 100 µL of Buffer AE was used instead of 200 µL, and (7) the QIAamp spin column was incubated at 22 °C for 20-25 min. after adding Buffer AE.

Table 2: Long range amplification primers

Primer name	Primer sequences (5'-3')	Amplicon length
299F	GGTCAATTTCGTGCCAGCCACCGCGGCCATACGATT	5578
5878R	GAGGAGTAGGAGGACTGCTGTAATGAAC	
5687F	GGGTGAACTGTTTATCCTCCTCTAGC	5803
11490R	GGTCCTATAATTACCTTGGGGCTCAG	
8497F	GTCATTATCTTACTAATTACCCTAGAATTAGGC	6465
14962R	GAATAGGAAGTATCATTCTGGTTTAATATGG	
14640F	CTCAGTAGACAAAGCCACCCTCACACG	2529
1018R	CCAAGCGCACTTTCCAGTACGCTTACC	

2.3 Amplification and sequencing

PCR amplification of mitochondrial DNA was carried out using a LongRange PCR Kit (Qiagen, Inc.), with a reaction volume of 25 µL and a reaction mix consisting of 2.5 µL of 10x LongRange PCR Buffer, 500 µM of each dNTP, 0.4 µM of each primer, 1 unit of Long-Range PCR Enzyme, and 50-150ng of template DNA. Cycling conditions were as follows: 93 °C for 3 min, followed by 50 cycles denaturing at 93 °C for 15 s, primer annealing at 53-58 °C (depending on primer set, with a decrease of 0.1 °C every cycle) for 30 s, and extension at 68 °C for 7 min, followed by 30 cycles of denaturing at 93 °C for 15 s, annealing at 48-53 °C (depending on primer set) for 30 s, and extension at 68 °C for 3.5 min (with an increase of 12 s every cycle), with a final extension at 68 °C for 7 min. To minimize the possibility of amplifying nuclear mitochondrial pseudogenes ("numts"), four sets of primers were used to generate overlapping amplicons from 2500 to 6000 bp in length, thereby enabling a quality test for genome circularity (Thalmann et al., 2004) (Table 2).

Both mtDNA strands were sequenced directly using BigDye Terminator v3.1 (Applied Biosystems, Inc.) and a suite of sequencing primers including primers previously designed for other platyrrhine mtDNA genomes (Hodgson et al., 2009), primers newly designed using mtDNA genomes available on GenBank, and primers newly designed by primer walking. All primer sequences are available upon request. Sequencing products were analyzed on an ABI 3730 DNA Analyzer system (Applied Biosystems, Inc.) and bases were called using Sequencing Analysis v5.2 (Applied Biosystems, Inc.). Base calls were verified by eye and sequences were assembled using the software Sequencher v4.7 (Gene Codes Corp.). Overlapping regions were examined for irregularities such as frameshift mutations and premature stop codons, with the lack of such irregularities indicating the absence of contaminating numt sequences.

2.4 Alignment

The seven newly generated *Saimiri* mtDNA genomes were added to a dataset of 21 primate mtDNA genomes available in GenBank, including two mtDNA genomes labeled as *"Saimiri sciureus"* (Table 3). The first (accession no. FJ785425) came from a Guyanese squirrel monkey (putative *S. s. sciureus*) from Three Springs Scientific (Perkasie, PA), while provenience information on the second (accession no. NC_012775) was unknown by the authors (Matsui, pers. comm.). Complete mtDNA sequences from 17 other haplorrhine primates were selected in order to provide nodes temporally constrained by well-supported fossil dates or to fill out major branches of the haplorrhine phylogenetic tree. Two strepsirrhine sequences

(*Lemur catta* and *Galago senegalensis*) were selected as outgroups.

It has been suggested that the 12 protein-coding genes encoded by the heavy strand of the mtDNA genome have the most similar evolutionary properties (e.g., Gissi et al., 2000); for this reason, we created a master dataset including only those gene sequences. Since homology is best identified at the amino acid level due to the arrangement of coding DNA into nucleotide triplets and the redundancy of the genetic code, each protein-coding gene was aligned based on its corresponding amino acid translations using the software TranslatorX (Abascal et al., 2010). We allowed the software to automatically identify the most likely reading frame (i.e. minimizing number of stop codons), then performed a protein alignment using the software Muscle (Edgar, 2004). Alignments were evaluated by eye using MacClade v4.08 (Maddison and Maddison, 2005). Next, the 12 individual gene alignments were concatenated using the software SequenceMatrix v1.7.6 (Vaidya et al., 2011) to create a master alignment of 10,836 bp total, equivalent to roughly 65% of the mtDNA genome.

2.5 Choice of nucleotide substitution model and phylogenetic tree inference

Phylogenetic trees were inferred using maximum likelihood and Bayesian inference approaches. Maximum likelihood analyses were run in RAxML v.7.2.6 (Stamatakis, 2006). To select the best-fitting model, 50 independent iterations were run using three data partitions (codon 1, codon 2, codon 3) and model parameters were estimated independently for each partition. An additional 50 iterations were then run using two data partitions (codons 1+2 combined, codon 3). For each analysis, the GTR + G (general time reversible model with gamma-distributed rate variation) model was employed to search for the maximum likelihood tree and topologic support was estimated with 1000 bootstrap replicates using the GTRGAMMA (Stamatakis, 2006).

Bayesian inference analyses were run in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) using a nucleotide substitution model based on that selected by MrModeltest v2.3 (Nylander, 2004). Data were partitioned by codon position and model parameters were estimated independently for each partition. Four independent Markov Chain Monte Carlo (MCMC) iterations were run for 10 million generations sampled every 1000 generations. Convergence was visually assessed using Tracer v1.5, plotting the likelihood versus generation and estimating the effective sample size (ESS >200) of all parameters across the four independent analyses. In addition, we used AWTY (Wilgenbusch et al., 2004) to plot pairwise split frequencies for the four independent MCMC runs and to check the posterior probabilities of clades for non-overlapping trees in the sample using the compare and slide commands, respectively. We then summarized the posterior distribution of trees by removing from each run 1 million generations as burn-in (10% of total generations). The results from different runs were combined using LogCombiner and TreeAnnotator v1.5.4 (Drummond and Rambaut, 2007).

2.6 Fossil calibrations and divergence date estimation

Fossil constraints were selected based on criteria for choosing appropriate calibration points, reviewed elsewhere (e.g., Raaum et al., 2005; Ho and Phillips, 2009). Hodgson et al. (2009)

Table 3: Sequences aligned in this study

Group	Taxon and identifier	Common name (Groves 2001)	Accession no.
Haplorrhini			
Anthropoidea			
Platyrrhini			
Cebidae	*Saimiri sciureus*	Common squirrel monkey	NC_012775
	Saimiri sciureus sciureus 1	Common squirrel monkey	FJ785425
	Saimiri sciureus sciureus 2	Common squirrel monkey	HQ644334[a]
	Saimiri sciureus macrodon	Common squirrel monkey	HQ644338[a]
	Saimiri boliviensis boliviensis 1	Black-capped squirrel monkey	HQ644339[a]
	Saimiri boliviensis peruviensis	Black-capped squirrel monkey	HQ644340[a]
	Saimiri oerstedii oerstedii	Central American squirrel monkey	HQ644337[a]
	Saimiri oerstedii citrinellus 1	Central American squirrel monkey	HQ644336[a]
	Saimiri oerstedii citrinellus 2	Central American squirrel monkey	HQ644335[a]
	Cebus albifrons	White-fronted capuchin	AJ309866
	Aotus lemurinus	Gray-bellied night monkey	FJ785421
	Saguinus oedipus	Cottontop tamarin	FJ785424
Atelidae	*Ateles belzebuth*	White-fronted spider monkey	FJ785422
Pitheciidae	*Callicebus donacophilus*	White-eared titi	FJ785423
Catarrhini			
Cercopithecoidea	*Chlorocebus aethiops*	Grivet	NC_007009
	Papio hamadryas	Hamadryas baboon	NC_001992
	Theropithecus gelada	Gelada	FJ785426
	Macaca mulatta	Rhesus monkey	AY612638
	Colobus guereza	Mantled guereza	AY863427
	Trachypithecus obscurus	Dusky leaf monkey	NC_006900
Hominoidea	*Homo sapiens*	Human	EF061150
	Pan troglodytes	Common chimpanzee	EU095335
	Gorilla gorilla	Western gorilla	D38114
	Pongo abelii	Sumatran orangutan	X97707
	Hylobates lar	White-handed gibbon	NC_002082
Tarsioidea	*Tarsius bacanus*	Western tarsier	NC_002811
Strepsirrhini			
Lemuriformes	*Lemur catta*	Ring-tailed lemur	AJ421451
Lorisiformes	*Galago senegalensis*	Senegal bushbaby	AB371092

[a] New sequences generated for this study

identified seven useful fossil constraints for dating platyrrhine divergences using these criteria. We applied six out of these seven calibrations (Table 4). In contrast to the study of Hodgson et al. (2009), however, two independent methods were employed to estimate divergence dates: the autocorrelated Bayesian method of Thorne and Kishino (Thorne et al., 1998; Thorne and Kishino, 2002) as implemented with multidivtime from the MULTIDISTRIBUTE package and the uncorrelated Bayesian relaxed-clock method of Drummond and Rambaut (2007) as implemented with BEAST v1.5.4.

Bayesian priors for multidivtime were chosen according to a procedure previously followed by Hodgson et al. (2009) based on recommendations in the multidivtime manual (Thorne et al., 1998; Thorne and Kishino, 2002). The root-to-tip mean and its standard deviation were set to 75 MYA. Fossil calibration intervals were specified according to the limits specified in Table 4. The evolutionary rate at the root of the tree and its standard deviation were set to the median root-to-tip branch length divided by the root-to-tip mean (75 MYA), or about 0.12. The autocorrelation parameter (brownmean) and its standard deviation were set to 2 divided by the root-to-tip mean (75 MYA), or about 0.026. After a burn-in period of 100,000 generations, MCMC chains were sampled every 100 generations until 10,000 samples were taken.

BEAUTi v1.5.4 was implemented to set priors and to prepare a file for use with BEAST v1.5.4 (Drummond and Rambaut, 2007). Instead of setting minimum and maximum limits, calibration points were implemented as log-normal distributions with an offset, mean, and standard deviation (0.5 in all cases) such that 95% of the prior distribution lay between the boundaries specified in Table 4. For two calibration points (*Aotus-Saguinus* and *Cebus-Saimiri*), an exponential distribution with a predefined offset (minimum bound) of 12.1 was

Table 4: Evolutionary rate calibration constraints (in millions of years)

	Divergence	Min.	Max.[a]	Fossil	References	Age
1.	*Homo/Pan*	5.0	8.0	*Ardipithecus*	Haile-Selassie (2001)	5.2
				Orrorin	Senut et al. (2001)	6.0
				Sahelanthropus	Vignaud et al. (2002) and Brunet et al. (2002)	6.0-7.0
2.	*Homo/Pongo*	12.5	18.0	*Sivapithecus*	Kelley (2002)	≈12.5
3.	Hominoid/cercopithecoid	21.0	30.0	*Morotopithecus*	Young and MacLatchy (2004)	>20.6
				Victoriapithecus	Pilbeam and Walker (1968)	≈19.0
					Benefit and McCrossin (2002)	
4.	*Papio/Theropithecus*	3.5	6.5	*Theropithecus*	Leakey (1993)	≈3.5
5.	*Aotus/Saguinus*	12.1	NA[b]	*Aotus dindensis*	Setoguchi and Rosenberger (1987)	≈12.1
6.	*Saimiri/Cebus*	12.1	NA[b]	*Neosaimiri*	Hartwig and Meldrum (2002)	≈12.1

[a] Maximum constraints were treated as hard boundaries in multidivtime. In BEAST, they were treated as the 95% limit of a log-normal distribution.

[b] Two calibrations were implemented as exponential distributions in BEAST with offset set to the minimum limit.

used. In contrast to the analysis run in multidivtime, the use of soft maximum bounds allowed for uncertainty in the older limits, which are inherently unknowable based on fossil evidence. In BEAST, eight independent iterations were run using three data partitions (codon 1, codon 2, codon 3) with six MCMC chains sampled every 1000 generations for 20 million generations after a burn-in period of 5 million generations. After checking all runs for convergence using Tracer v1.5 and AWTY as reported above, results from the eight independent runs were combined using LogCombiner and TreeAnnotator v1.5.4 (Drummond and Rambaut, 2007).

3 Results

3.1 Phylogenetic relationships

A single mtDNA tree topology was inferred for all of the maximum likelihood (ML) and Bayesian inference (BI) analyses conducted. Bootstrap values in the ML tree were weaker on average than corresponding support values obtained in BI trees, as shown in Fig. 2. In both ML trees, however, only one nodethe node supporting a sister relationship between *Aotus* and *Saguinus* in the tree using two data partitions (codons 1+2 combined, codon 3)had less than 75% support. Within the genus *Saimiri*, all analyses provided high support for a single topology, with most nodes showing 100% bootstrap support in the ML tree and 1.00 posterior probability values in the BI tree.

Within *Saimiri*, the boliviensis group, including the sequence of unspecified provenience mined from GenBank (accession no. NC_012775), was sister to all other *Saimiri* sequences included in the analysis. A clade consisting of the three Central American squirrel monkeys (*S. oerstedii*) formed a sister relationship to a second clade consisting of the two Guyanese squirrel monkeys (*S. s. sciureus*), with the Ecuadorian squirrel monkey (*S. s. macrodon*) sister to both.

In order to test the position of the boliviensis group (critical for testing the divergence date of crown *Saimiri*) and to corroborate the geographic origin of captive individuals included in our study, we added to our complete mitochondrial genome alignment six single-gene (cytochrome *b*) sequences derived from the dataset of Lavergne et al. (2010) (accession nos. AJ315388, EU232695, EU232701, EU232708, EU232710, EU232711), including four
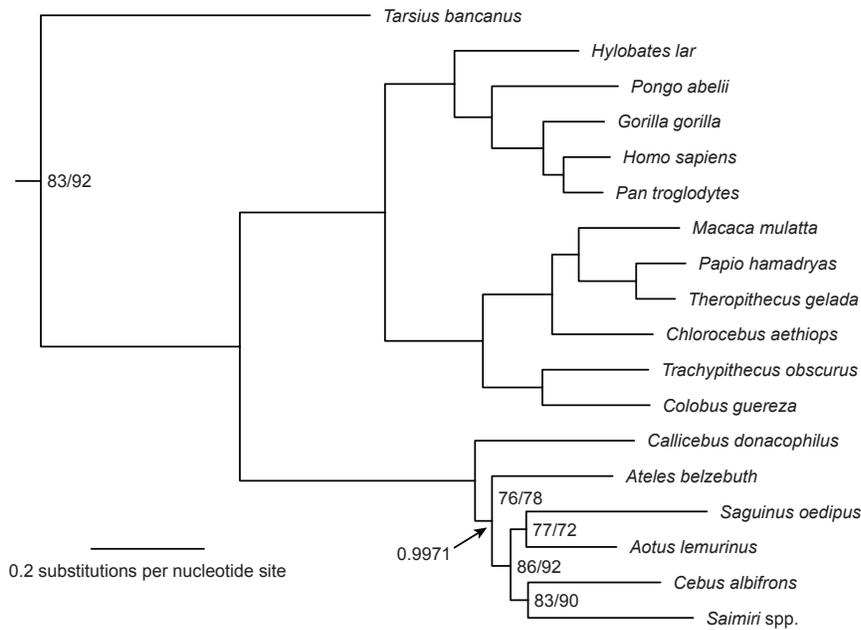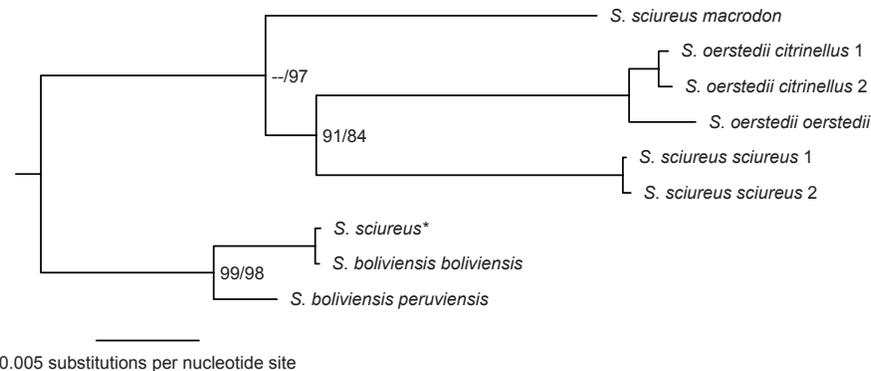
A

B

Figure 2: Single phylogenetic tree inferred from complete mitochondrial genome sequences based on maximum likelihood and Bayesian analyses. The tree topology remained constant across all analyses and branch lengths presented here follow the Bayesian analysis. The tree was rooted using *Lemur catta* and *Galago senegalensis* (not shown). Maximum likelihood values <100% are shown to the right of each node for the analyses using three data partitions (codon 1, codon 2, codon 3) and using two data partitions (codons 1+2 combined, codon 3), respectively. The single Bayesian posterior probability value <1.0 is indicated to the left of the node. For clarity, all *Saimiri* taxa are collapsed into one branch in (A) and magnified in (B). * denotes a sequence with unspecified provenience obtained from GenBank and assigned to *S. sciureus sensu lato*. Based on our analyses, we suggest it represents *S. b. boliviensis* (see text).

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Table 5: Comparison of Bayesian divergence estimates for major nodes in this study (in millions of years)

Divergence	multidivtime		BEAST	
	Mean	95% HPD	Mean	95% HPD
Cebus/Saimiri	15.39	12.42-20.37	13.82	12.10-16.09
Crown *Saimiri*	1.10	0.72-1.88	1.50	1.12-1.90
S. s. macrodon/S. oerstedii + S. s. sciureus	0.87	0.58-1.45	1.06	0.76-1.39
S. s. sciureus/S. oerstedii	0.77	0.51-1.28	0.91	0.63-1.21
S. o. oerstedii/S. o. citrinellus	0.11	0.06-0.18	0.16	0.09-0.23
S. b. boliviensis/S. b. peruviensis	0.25	0.14-0.52	0.30	0.17-0.44

taxa not included in our original alignment (one species: *S. ustus*; and three subspecies of *S. sciureus*: *S. s. cassiquiarensis*, *S. s. albigena*, and *S. s. collinsi*). Phylogenetic analyses run with both ML and BI did not alter the tree topology reported above and supported both a division of *S. boliviensis* from all other *Saimiri* and the taxonomy of samples of captive origin assigned a priori (results not shown).

3.2   Date estimates

The divergence date estimates obtained via the two methods did not differ substantially, with all median dates fitting into the 95% highest posterior densities (HPD) estimated from both analyses. Estimates for major nodes within *Saimiri* and between *Cebus* and *Saimiri* are presented in Table 5, along with 95% HPD intervals. Mean node ages for all other taxa are depicted in Fig. 3. In general, the estimates from BEAST tended to be slightly older than those obtained with multidivtime. This result can be attributed to the different implementations of maximum bounds in the analyses: hard bounds in multidivtime and soft bounds in BEAST. Here, the dates estimated from BEAST are discussed because the uncorrelated relaxed-clock method that it followed did not enforce strict calibration limits (Ho and Phillips, 2009).

4   Discussion

In this study, we provide an independent assessment of squirrel monkey phylogenetic relationships and divergence dates using complete mitochondrial DNA sequence data. With the exception of *S. sciureus*, all major species described by Hershkovitz (1984) and included in our analyses were monophyletic, in agreement with Lavergne et al. (2010). For four taxa not included in our complete mitochondrial sequence alignment (*S. ustus*, *S. s. cassiquiarensis*, *S. s. albigena*, and *S. s. collinsi*), we replicated the relationships described by Lavergne et al. (2010) by incorporating into our dataset mitochondrial cytochrome *b* sequences generated in their study (results not shown). All four taxa grouped with *S. s. macrodon*. While Lavergne et al. (2010) tentatively classified this group under the species *S. ustus* (Geoffroy Saint-Hilaire, 1843), we note that *Saimiri cassiquiarensis* (Lesson, 1840) was described 3 years earlier and would therefore be the valid taxon name for this species group.

Where previous genetic studies failed to conclusively resolve relationships among major *Saimiri* clades (Boinski and Cropp, 1999; Cropp and Boinski, 2000; Lavergne et al.,
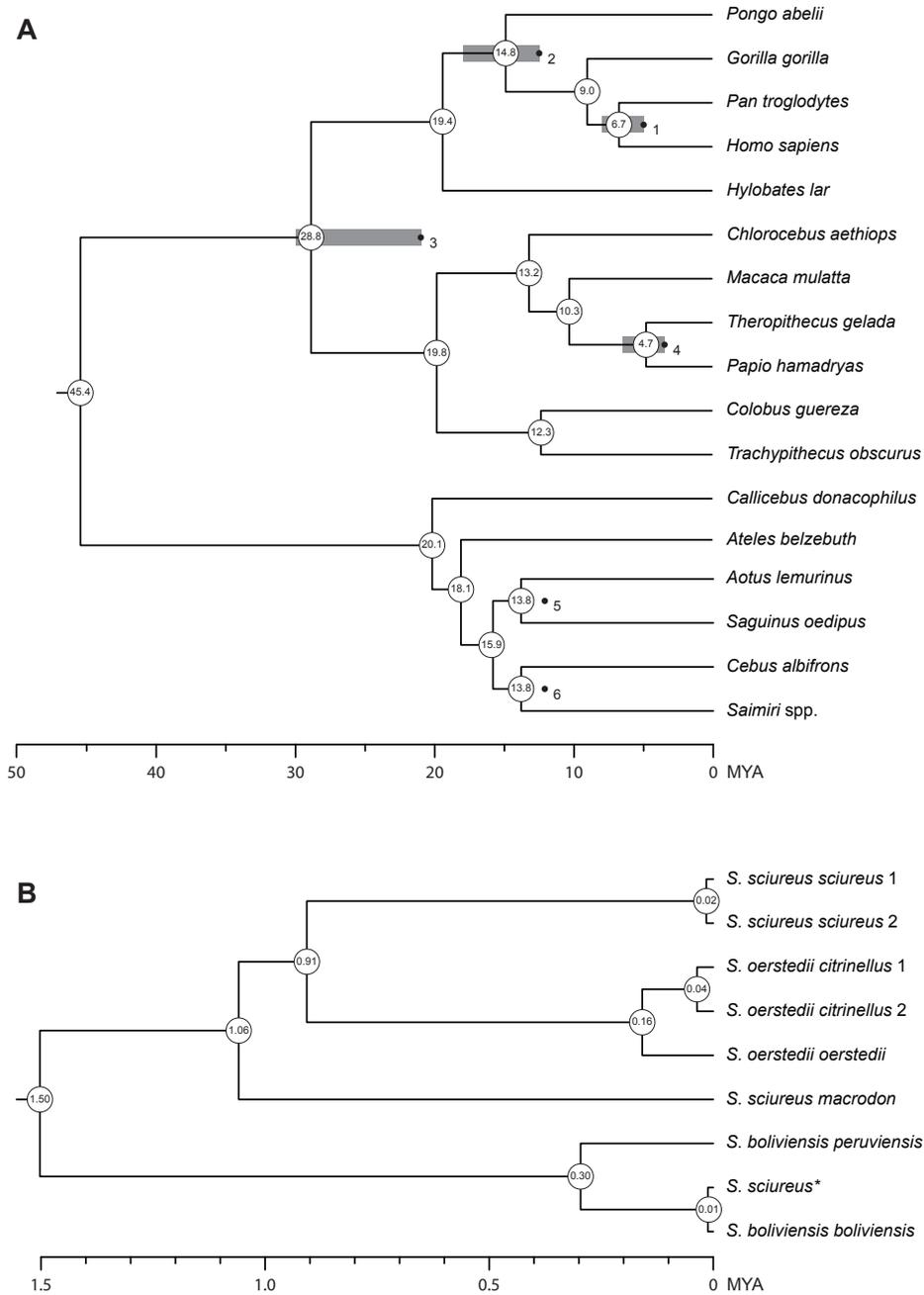
A

- *Pongo abelii*
- *Gorilla gorilla*
- *Pan troglodytes*
- *Homo sapiens*
- *Hylobates lar*
- *Chlorocebus aethiops*
- *Macaca mulatta*
- *Theropithecus gelada*
- *Papio hamadryas*
- *Colobus guereza*
- *Trachypithecus obscurus*
- *Callicebus donacophilus*
- *Ateles belzebuth*
- *Aotus lemurinus*
- *Saguinus oedipus*
- *Cebus albifrons*
- *Saimiri* spp.

50 40 30 20 10 0 MYA

B

- *S. sciureus sciureus* 1
- *S. sciureus sciureus* 2
- *S. oerstedii citrinellus* 1
- *S. oerstedii citrinellus* 2
- *S. oerstedii oerstedii*
- *S. sciureus macrodon*
- *S. boliviensis peruviensis*
- *S. sciureus**
- *S. boliviensis boliviensis*

1.5 1.0 0.5 0 MYA

Figure 3: Single chronogram with divergence date estimates from complete mitochondrial genome sequences. Because results from multidivtime and BEAST did not differ substantially, only results from BEAST are depicted here. Each calibration point (see Table 4) was implemented as an offset (black dot) with either a soft upper bound encompassing 95% of the prior log-normal distribution (gray rectangles) or no specified upper constraint. Mean node ages are depicted in circles. 95% HPD intervals for major nodes are presented in Table 5. The tree was rooted using *Lemur catta* and *Galago senegalensis* (not shown). *Tarsius bancanus* was allowed to group with the two outgroup taxa and is not shown here. For clarity, all *Saimiri* taxa are collapsed into one branch in (A) and magnified in (B). ∗ denotes a sequence with unspecified provenience obtained from GenBank and assigned to *S. sciureus sensu lato*. Based on our analyses, we suggest it represents *S. b. boliviensis* (see text).

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2010), our analyses strongly supported the position of *S. boliviensis* as sister to all other *Saimiri*. We also found strong support for a sister relationship between *S. s. sciureus* and *S. oerstedii*, with *S. s. macrodon* sister to this clade. These latter relationships, however, should be considered tentative in the absence of complete mitochondrial sequence data from *S. s. cassiquiarensis*, *S. s. albigena*, and *S. ustus*. At present, it is also unclear whether the paraphyly of *S. sciureus* in our mitochondrial tree and in the study of Lavergne et al. (2010) reflects true phylogenetic relationships, the effects of incomplete lineage sorting, or past episodes of interspecies hybridization. Additional markers from the nuclear genome are needed in order to address these issues. While one mined GenBank sequence (accession no. NC_012775) was labeled "*S. sciureus*," its strongly supported relationship with *S. b. boliviensis*, the lack of background information for the sample (Matsui, pers. comm.), and the tendency for captive squirrel monkeys to be designated as *S. sciureus* without substantiation (Boinski and Cropp, 1999) leads us to conclude that it represents an individual with an *S. b. boliviensis* mitochondrial haplotype, thereby preserving the monophyly of the *S. boliviensis* group.

Within squirrel monkeys, our estimated divergence dates differ substantially from previously published dates, with all mean dates and nearly all 95% highest posterior densities postdating the 1.8 MYA Vrica boundary marking the beginning of the Calabrian Stage, which until recently also designated the beginning of the Pleistocene Epoch. The recent ratification of the base of the Gelasian Stage, calibrated at 2.58 MYA, as the start of the Pleistocene (Gibbard et al., 2010) places all of our mean dates and credibility intervals well within the Pleistocene. Even using the younger boundary, which predominates the paleontological, biogeographical, and bioclimatological literatures to date, our results indicate that the timeline of mitochondrial diversification in extant squirrel monkeys falls firmly within the Pleistocene, not the Pliocene as argued elsewhere (Lavergne et al., 2010).

The Pleistocene timeline of squirrel monkey diversification indicates that Andean vicariance was unlikely to have driven speciation in extant squirrel monkeys. Precursors of Central American squirrel monkeys were therefore unlikely to have been present in northern Colombia and available to expand northward following the completion of the Isthmus of Panama (∼3.5 MYA), as has been argued by Ford (2006). In agreement with Cropp and Boinski (2000), our Central/South American squirrel monkey divergence of 0.9 MYA (95% HPD 0.6-1.2 MYA) predates estimates for the arrival of humans in South America, which range from 11,000 to 45,000 years ago (Dillehay, 1999). It is premature, however, to conclude that the presence of geographically isolated squirrel monkeys in Central America cannot be due to human transfer, as proposed by Hershkovitz (1969). In cases where population genetic diversities are not known, genetic divergence date inferences are necessarily older than actual population divergences (Arbogast et al., 2002); therefore, both our dataset and that of Cropp and Boinski (2000) are agnostic on this "artificial introduction hypothesis." If squirrel monkeys were introduced by humans to Central America, shared haplotypes should exist between Central and South American squirrel monkeys. The hypothesis of human transport is best evaluated by testing this prediction using population-level genetic data. At present, we note that the distribution of Central American squirrel monkeys continues to constitute a puzzling biogeographic problem in need of further study.

In South America, speciation in squirrel monkeys is considerably more recent than in larger primates, including spider monkeys (Collins and Dubach, 2000) and howler monkeys

(Cortés-Ortiz et al., 2003). In fact, the timing of squirrel monkey diversification falls even more recently than intraspecific patterns found in a single species of capuchin monkey (Ruiz-Garcia et al., 2010), a larger but similarly generalist primate (Janson and Boinski, 1992). In contrast to speciation events in most rainforest vertebrates (Moritz et al., 2000), the timing of mitochondrial diversification among extant squirrel monkeys is consistent with predictions made by the Pleistocene refuge hypothesis. The joining of rainforest patches during the Bramertonian interglacial period (1.55-1.3 MYA) corresponds with the initial divergence between *S. boliviensis* and all other squirrel monkeys (1.5 MYA) and may have enabled the expansion of precursors of *S. s. macrodon*, *S. s. sciureus*, and *S. oerstedii* northward from the southern Amazon Basin. Subsequently, forest contractions during the Pre-Pastonian glacial period (1.30-0.80 MYA) may have isolated *S. boliviensis*, *S. s. macrodon*, *S. s. sciureus*, and possibly *S. oerstedii*, promoting their diversification. Later glacial periods likely prompted further separation of populations but, given the fast-moving and versatile nature of squirrel monkeys (Hershkovitz, 1984), such barriers were probably temporary and gave way to population admixture in subsequent interglacial periods.

A growing body of evidence suggests that the origin of high species diversity in Amazonia significantly predates the climatic changes of the Pleistocene (Hoorn et al., 2010). Our results, however, suggest that squirrel monkeys diversified in the Pleistocene and the role of climate change and refugias in this process cannot be ruled out. If climate change was indeed a driving force for diversification within the genus *Saimiri*, attention should shift toward aspects of squirrel monkey ecology that shed light on how Pleistocene refuges played a heightened role in squirrel monkey evolution. Squirrel monkeys are small, fast-moving, highly gregarious arboreal animals (e.g., Kinzey, 1997) for which even narrow gaps in canopy cover may carry especially high risks of predation (Boinski et al., 2003). Recent studies indicate that glacial-interglacial changes in the Pleistocene had a major impact on Amazonian forest vegetation, resulting in a near-complete compositional turnover between cycles (Cardenas et al., 2011). The observation that most rainforest taxa diversified prior to the Pleistocene may therefore serve to illustrate that grasslands did not effectively deter gene flow in tropical rainforest species, with squirrel monkeys as a possible notable exception.

Insights into the evolution of squirrel monkeys are currently impeded by the paucity of the platyrrhine fossil record. If the Middle Miocene fossil *Neosaimiri* (∼12.5 MYA) indeed represents an ancestral form of *Saimiri*, given a crown diversification date of 1.5 MYA for extant squirrel monkeys, this leaves at least 11 million years of unobservable evolution. In agreement with Hodgson et al. (2009), the estimated divergence between *Cebus* and *Saimiri* at 13.8 MYA (12.1-16.1 MYA) indicates that the Early Miocene fossil *Dolichocebus* (∼20.5 MYA) cannot fall in the *Saimiri* lineage. Instead, *Dolichocebus* probably represents a stem platyrrhine and its morphological similarities with extant *Saimiri* (Rosenberger, 1979) reflect either convergence or plesiomorphy. The versatility of squirrel monkeys and their ability to colonize new areas rapidly indicates that squirrel monkeys may have been widely distributed at the start of the Pleistocene. Outside of the extant squirrel monkey lineage, however, any cladogenesis occurring between 12.5 and 1.5 MYA has since become extinct, with the rapid expansion of modern squirrel monkeys possibly indicating that a large portion of these extinctions occurred around the early Pleistocene.

At present, the degree to which diversification in extant squirrel monkeys has responded to climatic changes in the Pleistocene is unclear. Also unclear is the degree to which

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the observed genetic diversity in squirrel monkeys is a product of past ecological barriers and not a sampling artifact due to isolation by distance. For both questions, population-level genetic studies aimed at inferring the demographic histories of separate squirrel monkey populations have the potential for gleaning further insights into the biogeography of modern squirrel monkeys.

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