

Mesoamerican tree squirrels evolution (Rodentia: Sciuridae): a molecular phylogenetic analysis

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Abstract: The tribe Sciurini comprehends the genera *Sciurus*, *Syntheosciurus*, *Microsciurus*, *Tamiasciurus* and *Rheinthrosciurus*. The phylogenetic relationships within *Sciurus* have been only partially done, and the relationship between Mesoamerican species remains unsolved. The phylogenetic relationships of the Mesoamerican tree squirrels were examined using molecular data. Sequence data publicly available (12S, 16S, CYTB mitochondrial genes and IRBP nuclear gene) and cytochrome B gene sequences of four previously not sampled Mesoamerican *Sciurus* species were analyzed under a Bayesian multispecies coalescence model. Phylogenetic analysis of the multilocus data set showed the neotropical tree squirrels as a monophyletic clade. The genus *Sciurus* was paraphyletic due to the inclusion of *Microsciurus* species (*M. alfari* and *M. flaviventer*). The South American species *S. aestuans* and *S. stramineus* showed a sister taxa relationship. Single locus analysis based on the most compact and complete data set (i.e. CYTB gene sequences), supported the monophyly of the South American species and recovered a Mesoamerican clade including *S. aureogaster*, *S. granatensis* and *S. variegatoides*. These results corroborated previous findings based on cladistic analysis of cranial and post-cranial characters. Our data support a close relationship between Mesoamerican *Sciurus* species and a sister relationship with South American species, and corroborates previous findings in relation to the polyphyly of *Microsciurus* and *Syntheosciurus*' paraphyly. Rev. Biol. Trop. 62 (2): 649-657. Epub 2014 June 01.

Key words: phylogenetics, cytochrome B, *Sciurus*, *Microsciurus*, *Syntheosciurus*, Mesoamerica.

Tree squirrels are distributed throughout Eurasia and America, and grouped in the tribe Sciurini (Black, 1963; Wilson & Reeder, 2005). Sciurini comprehends the genera *Sciurus*, *Syntheosciurus*, *Microsciurus*, *Tamiasciurus* and *Rheinthrosciurus*. Among these, the genus *Sciurus*, with seven subgenera and 28 species (Wilson & Reader, 2005), is the most diverse and widespread, with representatives in Eurasia (*S. vulgaris*), the Japan Islands (*S. lis*), the Middle East (*S. anomalus*) and the Americas. In the Americas, 25 *Sciurus* species occur, with several species restricted to the Neartic region (*S. carolinensis*, *S. niger*, *S. aberti*, *S.*

griseus and *S. arizonensis*). The genus reaches its highest diversity in the Neotropical region with 20 species.

Among the Neotropical *Sciurus* species, *S. variegatoides*, *S. yucatanensis* and *S. richmondi* are restricted to the Mesoamerican region -the area between the Isthmus of Tehuantepec and the Isthmus of Panama-. Several other species (e.g. *S. deppei*, *S. granatensis*, and *S. colliaei*) occur in the Mesoamerican region albeit not exclusively. In addition to *Sciurus*, there are other Sciurini genera with Mesoamerican endemic taxa; the genus *Microsciurus* currently is composed by four species: *M. alfari* and *M.*



mimuls distributed in lower Central America, and *M. flaviventer* and *M. santandarensis* found in Northern South America. The genus *Syntheosciurus* presents only one species with two subspecies, *S. brochus poaensis* and *S. brocus brocus*, endemic of Talamanca-Chiriqui mountain range in Costa Rica and Panama.

The most complete taxonomic revision of the Sciurini was made by Moore (1959) based on the analysis of qualitative characters from the cranium. In this study, the genus *Sciurus* was placed in the subtribe Sciurina together with *Reinthrosciurus* Gray and *Guerlinguetus* Gray, which was later placed as a subgenus in *Sciurus* (Hall, 1981). An additional subtribe (Microsciurina) was also proposed for *Microsciurus* and *Syntheosciurus*. In relation to *Microsciurus*, the genus was described initially as a *Sciurus*' subgenus (Allen, 1895) and later elevated to full generic rank (Goldman, 1912). A similar situation occurred with *Syntheosciurus*, formerly considered a subgenus of *Sciurus* (Goodwin, 1943, 1946). These affinities between *Mesosciurus* (currently *S. granatensis*) and the species in *Microsciurus* were also suggested by Moore (1959).

The phylogenetic relationships within *Sciurus* have been only partially resolved. Several studies have corroborated the monophyly of *S. vulgaris* and *S. lis* (Oshida, Masuda, & Yoshida, 1996; Oshida & Masuda, 2000; Oshida, Arslan, & Noda, 2009). Also, previous analyses based on an incomplete taxon sampling have suggested that South American species might form a monophyletic clade (Hafner, Barkley & Chupasko, 1994; Steppan, Storz, & Hoffmann, 2004; Herron, Castoe, & Parkinson, 2004; Oshida et al., 2009). Moreover, the most species comprehensive phylogenetic study on neotropical tree squirrels to date (Villalobos & Cervantes, 2007) demonstrated the monophyly of the Mesoamerican squirrel species. In that study, Mesoamerican species were split in two clades including *S. aureogaster*, *S. colliaei*, *S. variegatoides* and *Syntheosciurus brochus*, and *S. richomondi*, *S. granatensis*, *S. deppei*, *S. yucatanensis* and *Microsciurus alfari*, respectively. These findings questioned

the validity *Syntheosciurus*, suggesting that *S. brochus* should be considered a *Sciurus* species, and also pointed to the possible polyphyly of *Microsciurus*.

The phylogenetic relationships within the Sciurini tribe have been the focus of several studies in recent years. An early study by Hafner et al. (1994) based on protein variation showed *Microsciurus flaviventer* in a *Sciurus* clade comprising *S. carolinensis*, *S. niger* and *S. stramineus*. The pioneering work of Mercer & Roth (2003), based on the mitochondrial (12S rDNA and 16S rDNA) and nuclear genes (IRBP), brought some information on the intra-tribe relationships and showed the paraphyletic nature of the *Sciurus* genera in relation to *Syntheosciurus* and *Microsciurus*, and the relationship of these genera with *Tamiasciurus*. These same results were recovered by Steppan et al. (2004) using two nuclear genes (RAG1 and *cmy*) and by Herron et al. (2004) using a mitochondrial gene (*cytb*). The close relationship between *M. flaviventer* and the South American *Sciurus* species is a common feature throughout these studies. Despite these relevant results, the relationships within *Sciurus* and its related genera remain largely unresolved. The current data information include several genes (nuclear and mitochondrial for a total of 8) but remains poor in the number of taxa included and represented, precluding the possibilities of a more comprehensive estimation of the phylogenetic relationships. A recent combined supermatrix approach using all gene sequence publicly available (Pečnerová & Martínková, 2012) also revealed the same relationships between taxa. However, the phylogenetic relationships between Mesoamerican species remained unresolved, only supporting with low probability the sister relationship between *M. alfari* and *S. granatensis*.

The methodological approach of concatenated supermatrix multilocus analysis has been recently point it as a possible source of error in phylogenetic estimation. First revealed by Maddison (1997), the phylogenetic trees depicted by particular gene histories not necessarily correspond with the species tree phylogeny due

to processes like horizontal transfer, lineage sorting, gene duplication and gene extinction. In conditions of short branches, closely related species and large population numbers the discrepancy between gene trees and species is expected to be more problematic (Maddison, 1997; Maddison & Knowles, 2006). Therefore, the use of several independent loci to estimate the species tree was suggested (Pamilo & Nei, 1998) and adopted as thought concatenation of several genes, as a common practice in phylogenetic reconstruction. However, recent studies into multigene phylogenetics have shown that concatenating genes can lead to poor estimation of species tree (Degnan & Rosenberg, 2006; Kubatko, 2007). When multiple loci are concatenate they are assumed to evolve according a single evolutionary tree, obviating the effect of recombination in decoupling the evolutionary histories of different loci (Dengnan & Rosenberg, 2006). Under simulated conditions

the supermatrix approach has shown a high probability of error estimating species trees, only 9 of 100 replicates contained the true species tree topology (Heled & Drummond, 2010).

In this study, we aim at resolving the phylogenetic relationships of the Mesoamerican tree squirrels using a Bayesian multispecies coalescence model. We provide new sequence data (CYTB) of four Mesoamerican *Sciurus* species and used the newly generated information together with published DNA sequences.

MATERIALS AND METHODS

Specimens analyzed: Twenty one taxa, representing all recognized genera New World Tree squirrels, were included in the phylogenetic analysis, (Table 1). The outgroup taxa included *Tamiasciurus hudsonicus*, *T. mearsi* and *Reithrosciurus macrotis*. More than one DNA sequences were included per

TABLE 1
Sciurini sequence data (Genbank accession numbers) used in the phylogenetic analysis

Species	12s	16s	CYTB	IRBP
<i>Microsciurus alfari</i>				AY227609
<i>Microsciurus flaviventer</i>	AY227549	AY227497	MFU46169	AY227610
<i>Reithrosciurus macrotis</i>	AY227550	AY227498		AY227611
<i>Sciurus aberti</i>			SAU10170, SAU10172	AY227612
<i>Sciurus aestuans</i>	J012746		AJ389530	FM162057
<i>Sciurus anomalus</i>			AB292675, AB292676	
<i>Sciurus aureogaster</i>			KC737847	
<i>Sciurus carolinensis</i>	JN113040, JN393212	DQ334839	FJ200744	
<i>Sciurus ignitus</i>				AY227615
<i>Sciurus deppei</i>			KC777298	
<i>Sciurus granatensis hoffmani</i>			KC758866	AY227613
<i>Sciurus griseus</i>				AY227614
<i>Sciurus lis</i>	D50286		AB192893, AB192907	
<i>Sciurus niger</i>	SNU59174, SNU67289	DQ334840	FJ200745, SNU10180	
<i>Sciurus stramineus</i>			AB292678, SSU46183	AY227618
<i>Sciurus variegatoides rigidus</i>			KC777296, KC777295	
<i>Sciurus variegatoides thomasi</i>			KC777297	
<i>Sciurus vulgaris</i>	AY227553, D50287	AY227501, DQ334841	AB030026, AB292681	AY227620
<i>Syntheosciurus brochus</i>	AY227563	AY227502		AY227626
<i>Tamiasciurus hudsonicus</i>	AY227555	AF147684, AY227504	FJ200700, FJ200731	AY227622, AF297281
<i>Tamiasciurus mearsi</i>			AF322954	

*novel DNA sequence data from this study.



species, depending on the availability in the GenBank database.

DNA extraction, amplification and sequencing: Genomic DNA was extracted from tissue (0.25g) or museum skin samples (50mm²) for six taxa (*S. aureogaster*, *S. deppei*, *S. granatensis hoffmani*, *S. variegatoides atrirufus*, *S. variegatoides rigidus* and *S. variegatoides thomasi*) using Wizzard Genomic Extraction Kit (Promega). A region of 1144bp from the mitochondrial cytochrome B gene was amplified using the primers H15910 and L14724 (Oshida, Liang-Kong, Masuda, & Yoshida, 2000). PCRs were set-up in a 25µL reaction volume containing the following reaction conditions: 1µL of template DNA, 2.5mm MgCl₂, 200µM dNTPs, 0.5pmol/µL of each primer and 1.25U of Taq DNA polymerase (Fermentas). The amplification profile consisted of an initial denaturalization step of 94°C for 4 min; followed by 40 cycles of amplification (94°C for 1min., 55°C for 1min, 72°C for 1min.) and a final extension at 72°C for 10min. PCR products were purified using Exo-sSap and sequenced in both directions on an ABI3130 automated Genetic Analyzer using BigDye 3.1 DNA sequencing chemistry (Applied Biosystems) and following manufacturer's instructions. Sequences from this study are available under GenBank Accession Nos. KC737847, KC758866, KC777295, KC777296, KC777297, KC777298.

Phylogenetic analysis: Contig assemblies for cytochrome B sequences of both strands were made with GENEIOUS 5.6.4 (Biomatters Ltd., Auckland, New Zealand). All sequences obtained for this study plus Genbank sequences were aligned with Muscle using the default parameters (Edgar, 2004). The molecular substitution model for each gene was selected using JModelTest (Guindon & Gascuel, 2003; Posada, 2008) and the Akaike information criteria (Burnham & Anderson, 2003); mitochondrial 12s and 16s rDNA were analyzed under the GTR+G model, CYTB under the GTR G+I

model; and the nuclear IRBP gene under the GTR+I model.

Multilocus phylogenetic reconstruction was performed using starBEAST (Heled & Drummond, 2010) for Bayesian inference, using the log-normal relaxed clock model with a Yule-prior for branch lengths. Two or more DNA sequences per taxa were considered when the information availability allows it, as recommended by Heled & Drummond (2010). Two independent runs for 100 million generations and sampled every 1 000 generations. The first 10 million generations were discarded as burn-in. TRACER v1.5 (Drummond & Rambaut, 2007) was used to estimate the effective sample size for each parameter (all resulting effective sample sizes exceeded 100) and calculate the mean and upper and lower bounds of the 95% highest posterior density interval (95% HPD). The trees sampled were combined using LogCombiner (Drummond & Rambaut, 2007) and resampled every 2 000 generations. Single locus phylogenetic reconstruction using the most complete dataset (i.e. cytochrome B) was performed using BEAST v1.7.4 (Drummond & Rambaut, 2007) and the same conditions as the multilocus approach, however only one run of 100 million generations, sampled every 1 000 generations, was used.

RESULTS

The four genes analyzed were variable in relation to the proportion of missing data, gap composition and number of species sampled (Table 2). The most complete data set was the cytochrome B gene followed by the nuclear gene IRBP. The proportion of sequences to taxa in the analysis was greater than 1.25 (12S rDNA) and up to 1.56 (CYTB). In total, the four loci included 4203bp. The implemented MCMC runs converged to a common stationary phase and the ESS for all estimated parameters was greater than 100.

The sister taxa relationship between *Tamiasciurus* and *Sciurus* was recovered with high support in our multilocus bayesian phylogeny (Fig. 1). The genus *Sciurus*

TABLE 2
Descriptive features of the each dataset analyzed

Gene	bp	Missing data (%)	gap (%)	Species	Sequences
12S	832	62.9	4.63	8	10
16S	1 049	70.9	9.72	7	9
CYTB	1 144	19.3	6.84	16	25
IRBP	1 178	58.1	1.91	12	13

Bp: base pairs.

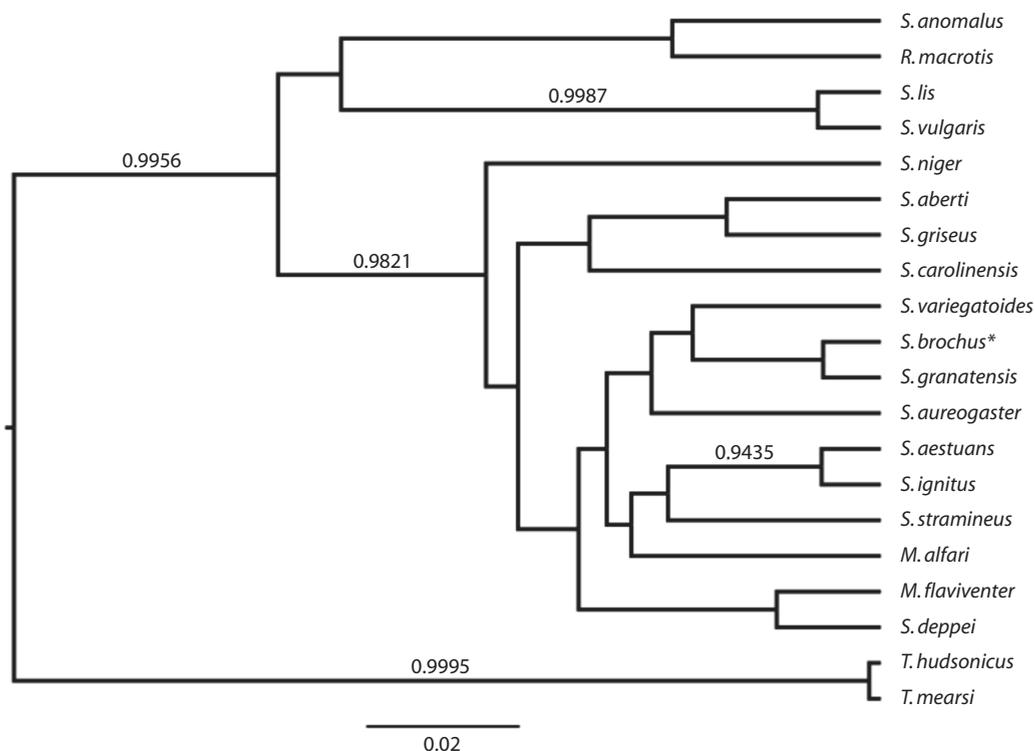


Fig. 1. Bayesian topology based on the multiple coalescence model derived from the multilocus analysis (CYTB, 12S and 16S mitochondrial genes, plus IRBP nuclear gene). Numbers above branches indicate the posterior probability (PP). No PP value is given for unsupported nodes (PP<0.85).

was paraphyletic including *Microsciurus* species (*M. alfari* and *M. flaviventer*). *Microsciurus* was not monophyletic. Also, none of the phylogenetic relationships previously reported within *Sciurus* were recovered with high posterior probability. The Eurasian species *S. vulgaris* and *S. lis* were monophyletic. Also, an unsupported relationship between *R. macrotis*,

S. anomalus (Middle East) and *S. vulgaris*-*S. lis* was recovered. The monophyletic nature of neotropical tree squirrels was corroborated but the internal relationships of this clade were unsupported. The only supported clade within this group includes *S. aestuans* and *S. ignitus*, both South American species, a relationship also recovered in previous studies.

The single locus analysis (Fig. 2) based on CYTB sequences, recovered similar phylogenetic relationships for the Eurasian *Sciurus* species. However, we found with strong support (PP=0.9989) the monophyly of all South American species included in the analysis (*M. flaviventer*, *S. stramineus* and *S. aestuans*). South American species were nested within a clade composed by all the mesoamerican species. In this clade, a monophyletic group containing *S. aureogaster*, *S. granatensis* and *S. variegatoides* was recovered (PP: 0.9976). *S. deppei* is placed in a polytomy with South American and Mesoamerica clades. In relation to nearctic species, *S. aberti* and *S. carolinensis* were placed them in a polytomy with the

Mesoamerican species, while *S. niger* was sister taxa to them.

DISCUSSION

Our multilocus analysis using the most complete molecular data set recovered the general relationships previously reported in the literature (Oshida & Masuda, 2000; Mercer & Roth, 2003; Pečnerová & Martínková, 2012), but failed to unveil novel phylogenetic relationships among *Sciurus* species with high posterior probabilities. This could be due to the missing data in the genes analyzed. Consequently, our current best estimation on the

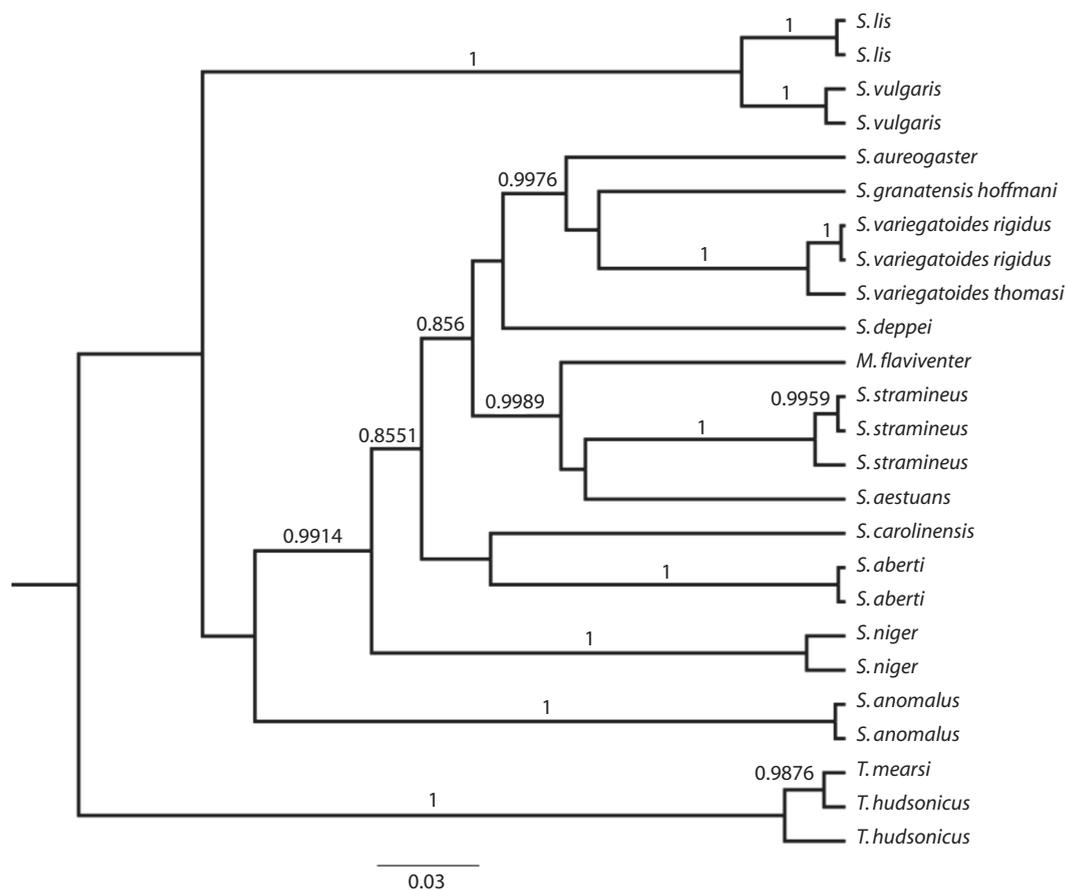


Fig. 2. Bayesian topology based on the multiple coalescence model derived from the CYTB gene sequences. Numbers above branches indicate the posterior probability (PP). no PP value is given for unsupported nodes (PP<0.85).

phylogenetic relationships of *Sciurus* species rest on the cytochrome B gene tree.

The single locus analysis shows a close relationship between *S. aureogaster*, *S. granatensis* and *S. variegatoides*, however, the relationships between these species remains unsolved. A close relationship between *S. aureogaster*, *S. variegatoides* and *S. colliaei* has been previously suggested based on phenotypic and distributional data (Musser, 1969), and a cladistic analysis of cranial and postcranial characters (Villalobos & Cervantes, 2007) within Sciurini/*Sciurus* found these species belonging to the same clade. However, this same analysis suggested that *S. granatensis* was part of a sister clade, which included *S. deppei*. The relationship between *S. deppei* and the *S. aureogaster* + *S. granatensis* + *S. variegatoides* clade cannot be rejected. The cytochrome b gene tree indicates that *S. deppei* is part of a polytomy, future sampling of more molecular characters is need to resolve it, and test the phylogenetic relationships obtained using morphological characters.

The South American clade recovered in previous studies (Mercer & Roth, 2003; Pečnerová & Martínková, 2012) was also found in the single locus analysis. The sister taxa relationship of *M. flaviventer*, *S. stramineus* and *S. aestuans* was reported by Pečnerová & Martínková (2012) based on the cytochrome B, cmy exons and IRBP gene trees, all with an adequate posterior probability. Our current analysis also suggests this relationship.

Based on the molecular clock analysis (Mercer & Roth, 2003) the divergence observed between the Mesoamerican species clade and the South American clade was estimated to take place 3.4 to 3.1 MYA. However, this speciation event may not be associated to the Panamian Isthmus closure as previously suggested (Mercer & Roth, 2003), but to the formation of savanna-like environments caused by Northern ice sheets formation (Molnar, 2008; Montes et al., 2012). Novel geologic mapping evidence in Central Panama suggest that the Panamian Isthmus was a continuous uninterrupted chain above sea level from the late Eocene to early

Miocene, with a closure at 15 MYA (Montes et al., 2012). The South America colonization and subsequent diversification may be expected to be a recent event. The central and Northern Andes deformation reach its current extension by late Oligocene to early Miocene (Oncken et al., 2006; Mora et al., 2010), providing a physiographic landscape similar to its present-day conformation. Therefore, the vicariant events associated to squirrel diversification in South America must be related to glacial cycles as has been suggested for the Mesoamerican *Sciurus* species (Villalobos, 2013).

The phenotypic variation observed among subspecies in some *Sciurus* may be associated to isolation events during glacial cycles (Moncrief et al., 2012). *S. variegatoides* comprises 15 subspecies with a high variation in color and coat pattern, distributed throughout Mesoamerica from the Tehuantepec Isthmus to the Panamian Isthmus (Harris, 1937; Hall, 1981). Seven subspecies are present and restricted to Costa Rica high and lowlands. Among these, *S. v. rigidus* and *S. v. thomasi* are related and allopatric: *S. v. rigidus* is distributed in Costa Rica's central valley highlands and into the Pacific coast, while *S. v. thomasi* is found mainly in the Caribbean coast and into the Talamanca Cordillera (Hall, 1981). The CYTB gene tree shows both subspecies as sister taxa, suggesting a potential phylogeographic pattern. These results contrast with the findings reported in *S. niger* subspecies (Moncrief, Lack & Van den Bussche, 2010; Moncrief et al., 2012). *S. niger* is also a highly diverse species containing 10 subspecies with a marked diversity in coat and color pattern. However, cytochrome B gene analysis has shown a lack of phylogeographic structure attributed to a rapid post glaciation range expansion and rapid phenotypic divergence (Moncrief et al., 2012). In order to evaluate the mechanism responsible for *S. variegatoides* subspecies diversity and to corroborate these preliminary results, a more extensive sampling is required.

Our data support a close relationship between Mesoamerican *Sciurus* species and a sister relationship with South American

species, both groups conform a larger clade distinct from the Neartic species. This study corroborates previous findings in relation to the polyphyly of *Microsciurus* and *Syntheosciurus*' paraphyly. Future studies remain necessary to clarify the phylogenetic position of the Mexican species of *Sciurus* and their relation to Mesoamerican species. Also, the diversification of South American species remains unresolved but centered in the Andes Cordillera as the region of endemism and speciation. At a lesser evolutionary scale, our data suggest the existence of phylogeographic structure within *S. variegatoides* subspecies, which deserves to be further analyzed.

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RESUMEN

Evolución de las ardillas arborícolas mesoamericanas (Rodentia: Sciuridae): un análisis filogenético molecular. La tribu Sciurini comprende los géneros *Sciurus*, *Syntheosciurus*, *Microsciurus*, *Tamiasciurus* y *Rheithrosciurus*. Las relaciones filogenéticas de *Sciurus* han sido resueltas parcialmente mientras que las relaciones de las especies Mesoamericanas permanecen sin resolverse. Las relaciones filogenéticas de las ardillas arborícolas mesoamericanas fueron estudiadas empleando datos moleculares. Datos de secuencias disponibles de forma pública (genes mitocondriales CYTB, 12S, 16S y gen nuclear IRBP) en conjunto con secuencias nuevas para el gen del Citocromo B de 4 especies mesoamericanas del género *Sciurus*, fueron analizadas empleando un modelo bayesiano de coalescencia multi-especie. Los análisis filogenéticos del conjunto de datos multilocus mostraron que las especies neotropicales forman un clado monofilético. El género *Sciurus* resultó ser parafilético debido a la inclusión de las especies de *Microsciurus* (*M. alfari* y *M. flaviventer*). Las especies suramericanas *S. aestuans* y *S. stramineus* presentaron una relación de especies hermanas. El análisis de un solo locus basado en el conjunto de datos más compacto y completo (secuencias del gen del citocromo B), apoyó la naturaleza monofilética de las especies suramericanas y recuperó un clado mesoamericano que

incluye a *S. aureogaster*, *S. granatensis* y *S. variegatoides*. Estos resultados corroboran los descubrimientos previos que emplearon datos morfológicos craneales y pos-craneales. Nuestros datos apoyan la relación cercana entre las especies de *Sciurus* Mesoamericanas y la relación hermana de estas con las especies de Suramérica, así como también corroboran la relación polifilética de *Microsciurus* y parafilética de *Syntheosciurus* previamente reportadas.

Palabras clave: filogenética, citocromo B, *Sciurus*, *Microsciurus*, *Syntheosciurus*, Mesoamerica.

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