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Molecular systematics of South American marsh rats of the genus *Holochilus* (Muroidea, Cricetidae, Sigmodontinae)

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We present a comprehensive systematic study of *Holochilus*, a sigmodontine genus of large, herbivorous, and semiaquatic rodents widely distributed in South America. Remarkably, given its complex taxonomic history and large economic as well as epidemiological importance, the alpha taxonomy of *Holochilus* has not benefited from a molecular-based approach. The study is based on sequences of 1 mitochondrial and 3 nuclear loci that were analyzed by maximum likelihood and Bayesian inference. Analyses include sequences of specimens from localities from Argentina, Bolivia, Brazil, Colombia, Paraguay, Peru, Suriname, and Uruguay, representing all but 2 of the species currently recognized in the genus. Of the 4 data matrices, the mitochondrial data set contains the largest geographic coverage and recovered 6 species-level lineages that form 2 well-supported species groups: the *brasiliensis* species group formed by *H. brasiliensis* and *H. vulpinus* and the *sciureus* species group composed by *H. chacarius*, *H. sciureus*, and 2 currently unnamed forms. Surprisingly, in the cytochrome *b* gene analyses, the 2 species groups are not sister to each other; i.e., *Holochilus* is not monophyletic, although these topologies lack significant support. However, the monophyly of *Holochilus* was supported by the 3 nuclear loci as well as by the combined analysis of all 4 loci. These genealogical results are the basis of taxonomic and biogeographic considerations.

Presentamos un estudio sistemático comprensivo sobre *Holochilus*, un género sigmodontino de grandes roedores herbívoros y semi-acuáticos ampliamente distribuido en América del Sur. Llamativamente, dada su compleja historia taxonómica y el gran impacto económico y epidemiológico, la taxonomía alfa de *Holochilus* no se ha beneficiado de un abordaje basado en evidencia molecular. El estudio se basa en secuencias de 1 gen mitocondrial y de 3 nucleares que fueron analizadas con máxima verosimilitud e inferencia Bayesiana. Los análisis incluyen secuencias de especímenes colectados en localidades de Argentina, Bolivia, Brasil, Colombia, Paraguay, Perú, Surinam y Uruguay, representando todas, con excepción de 2, las especies actualmente reconocidas en el género. La genealogía mitocondrial, que es la que tiene la mayor cobertura geográfica de *Holochilus*, recobra 6 linajes de nivel de especie que forman 2 grupos de especies bien apoyados: el grupo de especies *brasiliensis* integrado por *H. brasiliensis* y *H. vulpinus* y el grupo de especies *sciureus* que está compuesto por *H. chacarius*, *H. sciureus* y 2 formas aparentemente sin nominar. Llamativamente, en los análisis basados en el gen mitocondrial los 2 grupos de especies no son hermanos; i.e., *Holochilus* no es monofilético, aunque esta topología no tiene apoyo significativo. Sin embargo la monofilia de *Holochilus* es apoyada por los análisis de los 3 genes nucleares y por el análisis combinado de los 4 genes. Estos resultados genealógicos son la base de consideraciones taxonómicas y biogeográficas.

Key words: *Holochilomys*, Oryzomyini, Rodentia, South America, taxonomy

Marsh rats of the oryzomyine genus *Holochilus* Brandt, 1835 are large sigmodontine rodents that display several morphological specializations for an herbivorous diet and semiaquatic life (Hershkovitz 1955; Sierra de Soriano 1969; Massoia 1976). *Holochilus* is distributed from central Argentina and Uruguay in the south to northern Venezuela and the Guianas in the north and from the Amazonian regions of Bolivia, Peru, Ecuador, and Colombia to the Atlantic coast of eastern Brazil (Musser and Carleton 2005; Gonçalves et al. 2015). Across this large distribution, approximately 14 nominal forms are assigned to *Holochilus* as currently delimited (the fossil *primigenius* originally described as a form of *Holochilus* is now placed in *Reigomys*—see Machado et al. 2014). The history of these nominal forms is particularly complex. In brief, in addition to describing the new species *magnus* (now placed in its own genus *Lundomys* as a junior synonym of *L. molitor*—Voss and Carleton 1993), Hershkovitz (1955) placed all other forms in the synonymy of *H. brasiliensis* with *amazonicus*, *balnearum*, *berbicensis*, *brasiliensis*, *guianae*, *incarum*, *leucogaster*, *vulpinus*, *nanus*, and *venezuelae* as subspecies and *sciureus* and *chacarius* as junior synonyms of *H. brasiliensis brasiliensis*. Later, Massoia (1976, 1980) recognized 3 species (excluding *magnus*): *H. brasiliensis*, *H. chacarius*, and *H. sciureus*. Subsequent studies have suggested greater species diversity, revealing extensive interpopulation morphological and/or chromosomal variation (e.g., Freitas et al. 1983; Marques 1988; Aguilera and Pérez-Zapata 1989; Nachman and Myers 1989; Nachman 1992a, 1992b; Voss and Carleton 1993; Voglino et al. 2004; Brandão and Nascimento 2015). Given this evidence, some authors (e.g., Reig 1986; Aguilera et al. 1993) suggested that additional forms (e.g., *amazonicus*, *venezuelae*) be elevated to species level; however, the classification scheme proposed by Massoia (1976, 1980) has remained the most widely accepted during the intervening 3 decades (e.g., Musser and Carleton 2005). Recently, a new species, *H. lagigliai*, was described on the basis of material from west-central Argentina (Pardiñas et al. 2013). Finally, in what can be considered the first revisionary account of *Holochilus* since Hershkovitz (1955), Gonçalves et al. (2015), based on morphological and cytogenetic information, recognized 6 living species within the genus (*H. brasiliensis*, *H. chacarius*, *H. lagigliai*, *H. sciureus*, *H. venezuelae*, and *H. vulpinus*).

Remarkably, considering the extent of known morphological and chromosomal variation, the wide distribution of the genus, and its large economic and epidemiological importance (e.g., Rosa et al. 2005; Borda and Rea 2006; Eiris and Barreto 2009), taxonomic studies of *Holochilus* have not benefited from the use of molecular data. As such, we present here the 1st molecular-based systematic study centered on *Holochilus*. We analyzed DNA sequences of 1 mitochondrial and 3 nuclear loci. Our main goal is to generate a phylogenetic hypothesis for *Holochilus* that allows for the assessment of its contents and species boundaries.

Materials and Methods

Taxonomic and character sampling.—Animal care and use procedures followed guidelines approved by the American Society of Mammalogists (Sikes et al. 2011). Genealogical analysis was based on DNA sequences of 1 mitochondrial gene (cytochrome *b*: *Cytb*), 1 nuclear exon (interphotoreceptor retinoid binding protein, exon 1: *Rbp*3), and 2 nuclear introns (alcohol dehydrogenase, intron 2: *Adh*1-I2; beta-fibrinogen, intron 7: *Fgb*-I7). Sampling differs among loci, mostly due to difficulties with gene amplifications and lack of access to voucher specimens associated with GenBank sequences. The most exhaustive coverage is that of the *Cytb* matrix that includes sequences of 66 specimens belonging to 44 populations from Argentina, Bolivia, Brazil, Paraguay, Peru, Suriname, and Uruguay (Fig. 1; Table 1). This sample set includes specimens assignable to several of the nominal forms (e.g., *brasiliensis*, *chacarius*, *sciureus*, and *vulpinus*). However, we are lacking representatives of the recently described *H. lagigliai* (only known by its holotype, caught more than 60 years ago, and by few fossil remains) and *H. venezuelae* (however, see the discussion below). The *Rbp*3 matrix includes sequences of 20 specimens of *Holochilus* from 18 localities; the *Adh*1-I2 matrix encompasses sequences of 8 specimens of *Holochilus* collected at 8 localities; while the *Fgb*-I7 matrix includes sequences retrieved from 18 specimens of *Holochilus* gathered at 17 populations (Table 1). The 4 matrices also include sequences of the following genera that together with *Holochilus* are part of the so-called clade D of Oryzomyini (sensu Weksler 2006): *Aegialomys*, *Amphinectomys*, *Cerradomys*, *Eremoryzomys*, *Lundomys*, *Melanomys*, *Nectomys*, *Nesoryzomys*, *Oryzomys*, *Pseudoryzomys*, *Sigmodontomys*, and *Sooretamys* and 1 representative of the oryzomyine clade C, which is sister to Clade D (Weksler 2006), *Oligoryzomys*. Tree roots were placed on branches leading to *Oligoryzomys* (see sequence details in Supporting Information S1). Most of the specimens for whom sequences were gathered in this study were first identified on the basis of the direct inspection of their skulls or through the analysis of high-quality photos. Significant exceptions are 1 specimen here referred to *H. brasiliensis* (see below), whose skull cannot be found (B. K. Lim, Royal Ontario Museum, pers. comm.) and those here referred to *H.* sp. 1, whose sequences were downloaded from Genbank. Specimens for which sequences were gathered in this study are housed at the following collections: ASNHC: Angelo State Natural History Collection, San Angelo, Texas; BAL: Field number of Ulyses F. J. Pardiñas, to be deposited at the Museo de La Plata, La Plata, Argentina; CNP: Centro Nacional Patagónico, Puerto Madryn, Argentina; GD: field number of Guillermo D'Elía, to be deposited at Museo Nacional de Historia Natural, San Lorenzo, Paraguay; JPJ: field number of J. Pablo Jayat, to be deposited at the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia," Buenos Aires, Argentina; MNHN: Museo Nacional de Historia Natural, Montevideo, Uruguay; MVZ: Museum of Vertebrate Zoology, Berkeley, California; NK: Museum of Southwestern Biology, Albuquerque, New Mexico; PPA: field number of Ulyses F. J. Pardiñas, to be deposited at Centro Nacional Patagónico, Puerto Madryn, Argentina; ROM: Royal Ontario Museum, Toronto, Canada; TK and TTU: Museum of Texas Tech University, Lubbock, Texas; and UMMZ: University of Michigan Museum of Zoology, Ann Arbor, Michigan.

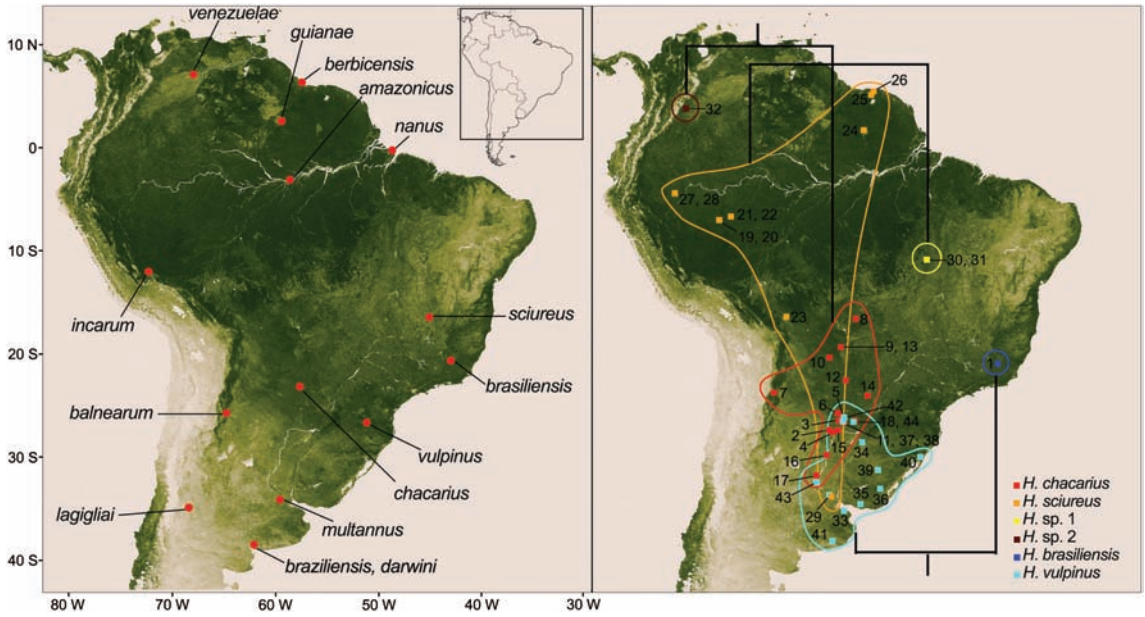

Fig. 1.—Right: map showing the type localities of taxa currently associated with *Holochilus*. Left: map of collecting localities of the specimens of *Holochilus* used in the present study; numbers correspond to those of Table 1. Polygons correspond to the species-level lineages identified in the phylogenetic analysis of *Cytb* gene sequences (see Fig. 2). Relationships among the species of each species group of *Holochilus*, as inferred on the basis of *Cytb* gene sequences (see text and Figure 2), are depicted with black lines.

DNA sequence acquisition.—The majority of DNA sequences analyzed were generated for this study; however, sequences archived in Genbank were also utilized (Supporting Information S1). The DNA sequences gathered in the present study were obtained according to the following protocol. Genomic DNA was isolated from approximately 0.1 g of liver or muscle tissue, using a Qiagen extraction kit (Qiagen, Inc., Valencia, California), Puregene DNA extraction kit (Gentra Systems, Minneapolis, Minnesota), or phenol–chloroform extraction (Longmire et al. 1997). DNA obtained from skin samples were obtained as above but with samples previously washed with a weak bleach solution and then 5 times with distilled water. Samples were incubated overnight in lysis buffer and proteinase K before being put into the reaction. These samples were processed in a biosafety hood that had been bleached and exposed to UV and using plastics that had been previously exposed to UV to minimize possibility of sample contamination. The complete *Cytb* gene (1,143 bp) was amplified by using polymerase chain reaction (PCR) with GoTaq (Promega, Madison, Wisconsin) and with primers MVZ05 (Smith and Patton 1993) and CB40 (Hanson and Bradley 2008) or L14115 and H15288 (Martin et al. 2000). Reaction concentrations (25 µl volume) included: ≤ 300 ng genomic DNA, 0.07 mM dNTPs, 2.86 mM MgCl, 2.5 µl 10× buffer, 0.75 U enzyme, and 0.286 µM of each primer. PCR thermal profiles included an initial denaturation at 95°C (2 min), 30–40 cycles with denaturation at 95°C (30 s), annealing 45°C (45 s), extension at 72°C (1 min 30 s), and a final extension cycle of 72°C (8 min). A fragment of *Rbp*3 (1,266 bp) was amplified with the same reaction concentrations as for the mitochondrial gene amplification, however was performed in 2 steps following Jansa and Voss (2000). Primers used in the 1st step were A1 and B2 (Jansa and Voss 2000), while primers used in the 2nd step were A1 and F (Jansa and Voss 2000) and E2 and B2 (Weksler 2003) for the downstream half. A fragment of the intron *Adh1*-I2 (ca. 534 bp) was amplified for using primers Exon II-F and 2340-II and the PCR conditions detailed in Amman et al. (2006). A fragment of the nuclear *Fgb*-I7 (approximately 605 bp) intron was amplified using primers Fgb-I7L-Rattus and Fgb-I7U-Rattus from Wickliffe et al. (2003). PCR conditions were those reported by Carroll and Bradley (2005).

Amplicons were purified by using the ExoSap PCR purification method (USB Corp., Cleveland, Ohio) then were sequenced using ABI Prism Big Dye Terminator v3.1 ready reaction mix (Applied Biosystems, Foster City, California). The primers used for PCR amplification were used together with internal primers (Mitochondrial: O400R, O700H [Hanson and Bradley 2008], F1 [Whiting et al. 2003], 752R, and 700L [Peppers and Bradley 2000]; *Rbp*3: C [Stanhope et al. 1992] and D2 [Weksler 2003]; Adh1-I2: 350F and 350R [Amman et al. 2006]; Fgb-I7: Bfib300F and Bfib300R [Carroll and Bradley 2005]) for cycle sequencing at 95°C (30 s) denaturing, 50°C (20 s) annealing, and 60°C (3 min) extension. Following 25–35 cycles, reactions were precipitated in isopropanol. Purified samples were sequenced using an ABI 3100-*Avant* automated sequencer. Sequencher 4.1 software (Gene Codes Corp. 2000) was used to proof nucleotide sequences (e.g., check for Numts) and chromatograms were examined to verify any discrepancies. Nuclear sequences showed low levels of heterogeneity and so no phasing analysis was performed; heterozygous sites were coded using the International Union of Biochemistry (IUB) polymorphic code (e.g., Y, R, M, C). Nucleotide sequences were deposited in GenBank (KP970125- KP970221).

Table 1.—List of specimens of *Holochilus* for which DNA sequences were analyzed in the present study. For details of which sequence was included for each specimen and the source of each sequence, see Supporting Information S1.

Species	Specimen number	Country	Division	Locality	Locality number
H. brasiliensis	ROM77592	Brazil	Minas Gerais	Viçosa	1
H. chacarius	CNP 1890	Argentina	Chaco	Selvas del Río de Oro	2
H. chacarius	CNP 1895	Argentina	Formosa	IPAF NEA, Laguna Blanca	3
H. chacarius	CNP 3937	Argentina	Chaco	4 km NW Puerto Las Palmas	4
H. chacarius	CNP 3941	Argentina	Chaco	4 km NW Puerto Las Palmas	4
H. chacarius	CNP 3942	Argentina	Formosa	IPAF NEA, Laguna Blanca	3
H. chacarius	CNP 3946	Argentina	Formosa	Río Bermejo, left bank	4
H. chacarius	CNP 3947	Argentina	Formosa	Puente Aº El Bellaco	6
H. chacarius	CNP 3953	Argentina	Chaco	Selvas del Río de Oro	2
H. chacarius	JPJ 1977	Argentina	Jujuy	Caimancito	7
H. chacarius	MVZ198012	Brazil	Mato Grosso	Base de Pesquisas Do Pantanal, CENAP/IBAMA	8
H. chacarius	TK129670	Paraguay	Alto Paraguay	Reserva Tres Gigantes	9
H. chacarius	TK61045	Paraguay	Alto Paraguay	Estancia Doña Julia	10
H. chacarius	TK61096	Paraguay	Alto Paraguay	Estancia Doña Julia	10
H. chacarius	TK61102	Paraguay	Alto Paraguay	Estancia Doña Julia	10
H. chacarius	TK61107	Paraguay	Alto Paraguay	Estancia Dona Julia	10
H. chacarius	TK61649	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK61650	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK61941	Paraguay	Pte. Hayes	Estancia Loma Pora	12
H. chacarius	TK62277	Paraguay	Alto Paraguay	Laguna Placenta	13
H. chacarius	TK62280	Paraguay	Alto Paraguay	Laguna Placenta	13
H. chacarius	TK64350	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK64396	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK64412	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK64425	Paraguay	Ñeembucú	Estancia Yacaré	11
H. chacarius	TK67332	Paraguay	Canindeyu	Reserva Mbaracayu, Jejui-mi	14
H. chacarius	UFES-CTA1539	Brazil	Mato Grosso	Base de Pesquisa do Pantanal, CENAP/IBAMA, 110 km SSW Poconé	8
H. chacarius	UFMG2999	Brazil	Mato Grosso	Base de Pesquisa do Pantanal, CENAP/IBAMA, 110 km SSW Poconé	8
H. chacarius	UFMG3000	Brazil	Mato Grosso	Base de Pesquisa do Pantanal, CENAP/IBAMA, 110 km SSW Poconé	8
H. chacarius	UMMZ166502	Argentina	Corrientes	0.5 km N of Itatí, island in rio Paraná	15
H. chacarius	UMMZ166519	Argentina	Corrientes	0.5 km N of Esquina, island in río Paraná	16
H. chacarius	UMMZ166525	Argentina	Santa Fe	12 km E Santa Fe, island in río Paraná	17
H. chacarius	UMMZ166526	Argentina	Santa Fe	12 km E Santa Fe, island in río Paraná	17
H. chacarius	UMMZ175065	Paraguay	Paraguari	1.2 km aguas abajo (N) orilla opuesta Hotel Centu Cué	18
H. chacarius	UMMZ175067	Paraguay	Paraguari	1.2 km aguas abajo (N) orilla opuesta Hotel Centu Cué	18
H. sciureus	MVZ190357	Brazil	Amazonas	near Miranda, río jurua	19
H. sciureus	MVZ193733	Brazil	Amazonas	Eirunepe, left bank, río Jurua	20
H. sciureus	MVZ193734	Brazil	Amazonas	Penedo, right bank, río Jurua	21
H. sciureus	MVZ193736	Brazil	Amazonas	Altamira, río Jurua	22
H. sciureus	NK102248	Bolivia	Santa Cruz	6 km W Ascensión de Guarayos	23
H. sciureus	TK10175	Suriname	Sipaliwini [a]	Sipaliwini Airstrip	24
H. sciureus	TK17512	Suriname	Paramaribo	Plantation Clevia	25
H. sciureus	TK17914	Suriname	Sipaliwini [a]	Sipaliwini Airstrip	24
H. sciureus	TK17917	Suriname	Sipaliwini [a]	Sipaliwini Airstrip	24
H. sciureus	TK21608	Suriname	Para	Zanderij	26
H. sciureus	TK53507	Peru	Loreto	Zona Marino	27
H. sciureus	TK53509	Peru	Loreto	Iquitos, Hospital Iquitos	28
H. sciureus	TTU76303	Peru	not recorded	not recorded	
H. sciureus	UMMZ166480	Argentina	Entre Ríos	6 km S Puerto Ibicuy	29
H. sp. 1	UFES-MAM1304	Brazil	Tocantins	Margem esquerda do río Javaés, Parque Estadual do Cantão	30
H. sp. 1	UFES-MAM1306	Brazil	Tocantins	Lagoa da Confusão, Fazenda Lago Verde	31
H. sp. 1	UFES-MAM1307	Brazil	Tocantins	Lagoa da Confusão, Fazenda Lago Verde	31
H. sp. 2	ROM90531	Colombia	Meta	Finca El Laqunazo, Vereda Memqua, Puerto Lopez	32
H. vulpinus	BAL00-05-15	Argentina	Buenos Aires	La Balandra	33
H. vulpinus	CNP 3965	Argentina	Corrientes	RP 94 y Aº Pariopá, Santo Tomé	34
H. vulpinus	MNHN5292	Uruguay	Canelones	Rincón del Colorado	35
H. vulpinus	MNHN5293	Uruguay	Canelones	Rincón del Colorado	35

Table 1.—*Continued*

Species	Specimen number	Country	Division	Locality	Locality number
H. vulpinus	MNHN6213	Uruguay	Treinta y Tres	Quebrada de Los Cuervos	36
H. vulpinus	GD071	Paraguay	Ñeembucu	Estancia Yacaré, 043 km SSW of Puesto San Fernando.	37
H. vulpinus	GD081	Paraguay	Ñeembucu	Estancia Santa Teresa, 2.95 km S of Puesto Anastacio	38
H. vulpinus	MCNU1943	Brazil	Rio Grande do Sul	Município de Dom Pedrito	39
H. vulpinus	MCNU1946	Brazil	Rio Grande do Sul	Município de Tramandaí	40
H. vulpinus	PPA589	Argentina	Buenos Aires	San José	41
H. vulpinus	TK66107	Paraguay	Ñeembucu	Estancia San José, 5 km E of house	42
H. vulpinus	UMMZ166477	Argentina	Entre Ríos	6 km S of Puerto Ibicuy	29
H. vulpinus	UMMZ166478	Argentina	Entre Ríos	6 km S of Puerto Ibicuy	29
H. vulpinus	UMMZ166524	Argentina	Entre Ríos	Las Cuevas, 35 km SSE Diamante	43
H. vulpinus	UMMZ166532	Argentina	Entre Ríos	Las Cuevas, 35 km SSE Diamante	43
H. vulpinus	UMMZ166533	Argentina	Entre Ríos	Las Cuevas, 35 km SSE Diamante	43
H. vulpinus	UMMZ175090	Paraguay	Misiones	620 m S Hotel Centu Cué	44
Outgroup					
Aegialomys galapagoensis	ASK4105	Ecuador	Galapagos	Isla Santa Fe	
Amphinectomys savamis	MV97005	Peru	Loreto	Iquitos, San Pedro	
Cerradomys scotti	TK61881	Paraguay	Canindeyú	Reserva Natural del Bosque Mbaracayú	
Eremoryzomys polius	FMNH129243	Peru	Amazonas	Balsas	
Lundomys molitor	EMG1779	Uruguay	Flores	Costas de Río San José	
L. molitor	MNHN4292	Uruguay	Colonia	Arroyo Cufré	
Melanomys chrysomelas	TTU100324	Nicaragua	Región Autonoma Atlántico Norte	El Balsamo	
Nectomys squamipes	TTU82920	Paraguay	Paraguari	Parque Nacional Ybicuí	
Nesoryzomys swarthi	ASNHC10003	Ecuador	Galápagos	Isla Santiago	
Oligoryzomys nigripes	MN78705/ MN62113	Brazil/ Brazil	Minas Gerais/ Rio Grande do Sul	Serra dos Vilela/Aratiba	
Oryzomys palustris	TTU82920	United States	Texas	Texas City	
Pseudoryzomys simplex	CNP 4589	Argentina	Chaco	4 km NW Puerto Las Palmas	
P. simplex	TK62425	Paraguay	Alto Paraguay	Estancia Tres Marías	
Sigmodontomys alfari	TTU 1033047	Ecuador	Esmeraldas	Estación Experimental "La Chiquita"	
Sooretamys angouya	TK61763	Paraguay	Ñeembucu	Estancia Yacaré	

[a] Specimens were collected before the creation of Sipaliwini district, as such their specimen label states Nickerie district.

Data analyses.—Sequence alignment was done with Clustal W as implemented in MEGA 6 (Tamura et al. 2013) using the default values for all alignment parameters. Adjustments by eye were only needed in the *Fgb*-I7 alignment. Phylogenetic analyses were carried out for each gene matrix separately. Each matrix was analyzed with 2 model-based methods: Bayesian inference (BI—Rannala and Yang 1996) and maximum likelihood (ML—Felsenstein 1981). Bayesian analyses were conducted using MrBayes 3.1 (Ronquist and Huelsenbeck 2003), with 2 independent runs, each with 3 heated and 1 cold Markov chains. The models used (*Cytb*: GTR + I + G; *Rbp*3: K80 + G; *Fgb*-I7: GTR + G; *Adh*1-I2: HKY + G) were selected with the Bayesian information criterion (Schwarz 1978) using jModelTest 2.1.2 (Guindon and Gascuel 2003; Darriba et al. 2012). All model parameters were estimated in MrBayes. Uniform-interval priors were assumed for all parameters except base composition and substitution model parameters, which assumed a Dirichlet prior. Runs were allowed to proceed for 20 million generations with trees sampled every 1,000 generations per chain. To check for convergence on a stable log-likelihood value, we plotted the log-likelihood values against generation time for each. The 1st 25% of the trees were discarded as burn-in and the remaining trees were used to compute a 50% majority rule consensus tree and obtain posterior probability (PP) estimates for each clade. ML analyses were conducted in Treefinder (Jobb et al. 2004). The best-fitting model of nucleotide substitution for each gene was selected with the Akaike information criterion (Akaike 1974) in Treefinder using the "propose model" routine: *Cytb*: J2 + G; *Rbp*3: TIM + G; *Fgb*-I7: GTR + G; *Adh*1-I2: TVM + G. J2 is a special case of the GTR model that includes 2 transversion parameters, one for transversions TA and CA and the other for transversions TG and CG. Except for this constraint, base frequencies, substitution rates, and gamma shape parameter were freely estimated from the data (Jobb 2011). We estimated the best tree under the model of nucleotide substitution previously selected using the search algorithm 2 as implemented in Treefinder; nodal support was estimated with 1,000 bootstrap pseudoreplicates (BS).

A matrix with all genes concatenated was analyzed as in the single-gene BI and ML analyses using the selected substitution model for each gene partition. This matrix was constructed with specimens for whom at least 2 genes were sequenced (Supporting Information S1); it also included the *Cytb* sequences of specimens ROM77592 and ROM90531 because these specimens are the single representatives of the species *H. brasiliensis* and *H.* sp. 2, respectively (see below). As such, the concatenated matrix includes 36 terminals of which 21 correspond to *Holochilus*. For those terminals lacking one or more

gene, the matrix was completed with ambiguous state characters (i.e., *n*). Finally, observed genetic distances (*p*-distances) were calculated in MEGA 6 (Tamura et al. 2013) using the *Cytb* dataset.

Results

The analysis of the *Cytb* matrix failed to recover a monophyletic *Holochilus* using both BI and ML approaches (Fig. 2; SM2). Marsh rat haplotypes fall in 2 well-supported, non-sister clades. One of these clades, that of the *brasiliensis* species group (PP = 1; BS = 94), is composed of 2 species-level lineages (sensu de Queiroz 1998). One of these lineages constitutes a highly supported clade (PP = 1; BS = 99) formed by haplotypes from specimens collected in central-eastern Argentina, southeastern Paraguay, and Uruguay and corresponds to the species *H. vulpinus*. This species shows a marked geographic structure with 2 allopatric main clades that are 3.3% divergent (Fig. 1). One of these clades (PP = 1; BS = 100) is distributed along the northern portion of the species range (localities 29, 34, 37, 38, 42, 43, and 44) in Paraguay and northwestern Argentina (Entre Ríos and Corrientes provinces). The other clade has no significant support (PP = 0.78; BS = 50) and in turn is formed by 2 allopatric subclades, the 1st (PP = 0.97; BS = 87) corresponding to 3 haplotypes from Uruguay (localities 35 and 36) and the other (PP = 1; BS = 91) is formed by the 2 analyzed haplotypes from the Argentinean province of Buenos Aires (localities 33 and 41; Fig. 2). The clade of *H. vulpinus* is sister to the 2nd species-level lineage of the *brasiliensis* species group, that of *H. brasiliensis*, which in our matrix is represented by a single haplotype obtained from a skin clip of a specimen (ROM77592) collected at Viçosa in the Brazilian state of Minas Gerais (locality 1; approximately 180 km SE Lagoa Santa, Minas Gerais, the type locality of *H. brasiliensis* according to the restriction made by Hershkovitz 1955). Based on the *Cytb* data (using both BI and ML approaches), the *brasiliensis* species group was determined to be sister to *Pseudoryzomys*, although this relationship lacks significant support (PP = 0.56; BS < 50). The clade formed by the *brasiliensis* species group and *Pseudoryzomys* is sister to the 2nd main clade of *Holochilus*.

The 2nd major clade of *Holochilus* haplotypes corresponds to the *sciureus* species group (PP = 1; BS = 100); within this clade, 4 species level lineages are recovered (Fig. 2; Supporting Information S2). Of these, the one with the largest distribution

Fig. 2.—Genealogical relationship of 66 haplotypes of the *Cytb* gene of the genus *Holochilus* recovered in the Bayesian analysis. Locality numbers are given next to specimen numbers and correspond to those of Table 1, Fig. 1, and Supporting Information S2. Support values correspond to posterior probability; within-species only values equal or above 0.95 are shown unless a given clade is discussed in the text. Maximum likelihood (ML) bootstrap proportions (only values above 50%) are shown, after the diagonal, for the main clades discussed in the text; for more details of the ML analysis (ln = −15401.9), see Supporting Information S1.

is that of *H. sciureus* (PP = 1; BS = 98), which occurs in a single locality in central-eastern Argentina (Entre Ríos province; locality 29), central Bolivia (Santa Cruz department; locality 23), northern Brazil (Amazonas state; localities 19–22), northeastern Peru (Loreto department, localities 27 and 28), and southern Suriname (Sipaliwini, Para and Suriname districts; localities 24–26). The 2nd lineage (PP = 1; BS = 99) is widely distributed in the Chaco, Pantanal, and ecotonal neighboring areas in central and northern Argentina (Chaco, Formosa, Jujuy, and Santa Fe provinces; localities 2–4, 6, 7, 15–17), eastern and western Paraguay (localities 9–14, 18), and south central Brazil (Mato Grosso state; locality 8) and corresponds to *H. chacarius*. Another lineage (PP = 1; BS = 100), which is here referred as *Holochilus* sp. 1, is represented by 2 haplotypes recovered from specimens (UFES-MAM1306, UFES-MAM1307) collected at Lagoa da Confusão in the central Brazilian state of Tocantins (locality 31; Figs. 1B and 2). The last of the 4 lineages is formed by 1 haplotype from a skin clip of a specimen (ROM90531) collected at Puerto López in the Colombian department of Meta (locality 32) and is here referred as *Holochilus* sp. 2 (Figs. 1B and 2). Relationships among these 4 lineages vary between analyses. The ML analysis (Supporting Information S2) recovers the following topology that lacks significant support: *sciureus* and sp. 2 form a clade sister to *chacarius*; sp. 1 is sister to the clade formed by the other 3 species. The Bayesian topology (Fig. 2) shows a polytomy at the base of the clade of the *sciureus* species group that involves the 4 species.

In summary, the *Cytb* matrix recovers 6 lineages that we recognize as putative species; 2 (*brasiliensis* and *vulpinus*) belong to the *brasiliensis* species group, and 4 (*chacarius*, *sciureus*, sp. 1, and sp. 2) belong to the *sciureus* species group (Figs. 1B and 2; Supporting Information S2). Observed *p* distances estimated using *Cytb* sequence data within and among the species-level clades identified above range from 0.000 (sp. 1) to 0.018 (*sciureus* and *vulpinus*) and from 0.067 (comparison *chacarius* versus sp. 2) to 0.147 (*sciureus* versus *vulpinus*), respectively (Table 2).

The 3 nuclear matrices independently recovered a monophyletic *Holochilus* (Fig. 3; Supporting Information S3); this clade is highly supported in the *Rbp*3 (PP = 1; BS = 99) and *Fgb*-I7 (PP = 1; BS = 100) data sets, but poorly supported based on the *Adh*1-I2 locus (PP = 0.74; BS = 63). Based on nuclear data, most relationships among alleles of *Holochilus* are poorly resolved and lack statistical support (Fig. 3; Supporting Information S3). Analyses of the *Rbp*3 matrix recover 2 main clades that do not match either species groups or species; in addition, recovered relationships are poorly supported. The topology derived from the *Fgb*-I7 matrix is the least resolved of all those gathered from nuclear genes; no species group or species is recovered as monophyletic. Analysis of the *Adh*1-I2 locus within the clade of *Holochilus* recovered a clade corresponding to the *brasiliensis* species group (PP = 1; BS = 100) that is sister to that of the *sciureus* species group (PP = 0.99; BS = 86); relationships within each of these clades are mostly unresolved and relationships do not recover the species-level clades found in the *Cytb* analysis.

Analyses of the concatenated matrix recovered a monophyletic *Holochilus* (PP = 1; BS = 93), which is sister (PP = 1; BS = 93) to *Pseudoryzomys*. The 4 species for which more than 1 representative was included, *H. chacarius*, *H. sciureus*, *H.* sp. 1, and *H. vulpinus*, were recovered as monophyletic and 3 exhibited high support, the exception being *vulpinus* (PP = 0.77; BS = 74). Similarly, the monophyly of the *brasiliensis* (PP = 1; BS = 80) and *sciureus* (PP = 1; BS = 95) species groups is also recovered; however, relationships among species of the *sciureus* species group lack significant support (Fig. 4; Supporting Information S4).

Table 2.—Observed genetic *p* distance of the cytochrome *b* gene within and among 6 species of *Holochilus*. Numbers in parentheses refer to the number of sequences studied for each species. n/a, not applicable.

	Intraspecific	Interspecific				
brasiliensis (1)	n/a					
vulpinus (15)	0.018	0.089				
chacarius (35)	0.010	0.132	0.136			
sciureus (13)	0.018	0.134	0.147	0.068		
sp. 1 (2)	0.000	0.132	0.137	0.078	0.082	
sp. 2 (1)	n/a	0.131	0.133	0.067	0.068	0.079

Discussion

After *Sigmodon* Say and Ord, 1825 and *Akodon* Meyen, 1833, *Holochilus* is the 3rd oldest generic name connected with the family Sigmodontinae as currently understood. In 180 years, the taxonomic history of the genus and its associated forms has proven to be not only highly complex but also unstable. In this regard, it is interesting to note that with the sole exception of *H. lagigliai*, which was recently described (Pardiñas et al. 2013), all other nominal forms of *Holochilus* were erected prior to 1953; in fact, most of them date to the 19th century and the 1st decade of the 20th century. Therefore, the distinct classificatory schemes presented in the last 7 decades, where 1 (Hershkovitz 1955—excluding *magnus*), 2 (Voss and Carleton 1993), 3 (Massoia 1980; Musser and Carleton 2005), 4 (Aguilera et al. 1993), 5 (Reig 1986), or 6 (Gonçalves et al. 2015; including the recently described *H. lagigliai*) species are recognized, reflect distinct visions on species limits. This disagreement is prompted by the difficulty in assessing the pattern of morphological variation in the genus and by extension the difficulty clarifying its alpha diversity. This fact in turns has its roots fundamentally in the lack of comprehensive studies of geographic variation of the genus. The present study is the first aimed to evaluate species limits within *Holochilus* on the basis of the analysis of DNA sequences. We analyzed patterns of variation of 4 loci, 3 nuclear and 1 mitochondrial. Results shed light on different issues related to the taxonomy of *Holochilus*, these range from the limits and contents of the genus, to species groups and species limits. The last 2 issues are discussed mostly in light of the *Cytb*-based analyses given that sampling of nuclear DNA sequences is reduced and nuclear topologies do not show resolution within *Holochilus*.

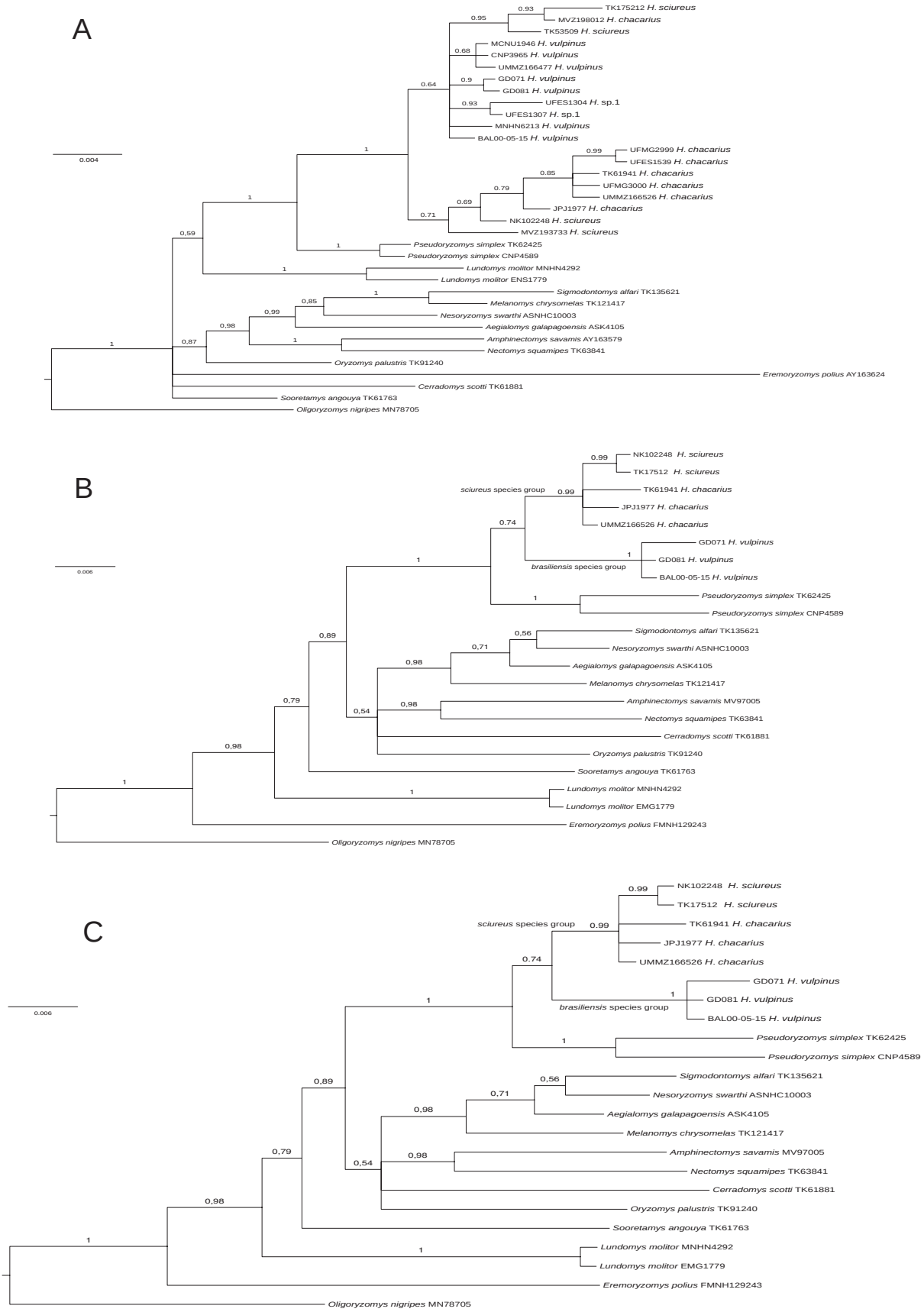

Fig. 3.—Genealogical relationship of DNA sequences of the loci A) *Rbp*3, B) *Adh*1-I2, and C) *Fgb*-I7 of the genus *Holochilus* recovered in the Bayesian analysis. Support values correspond to posterior probability.

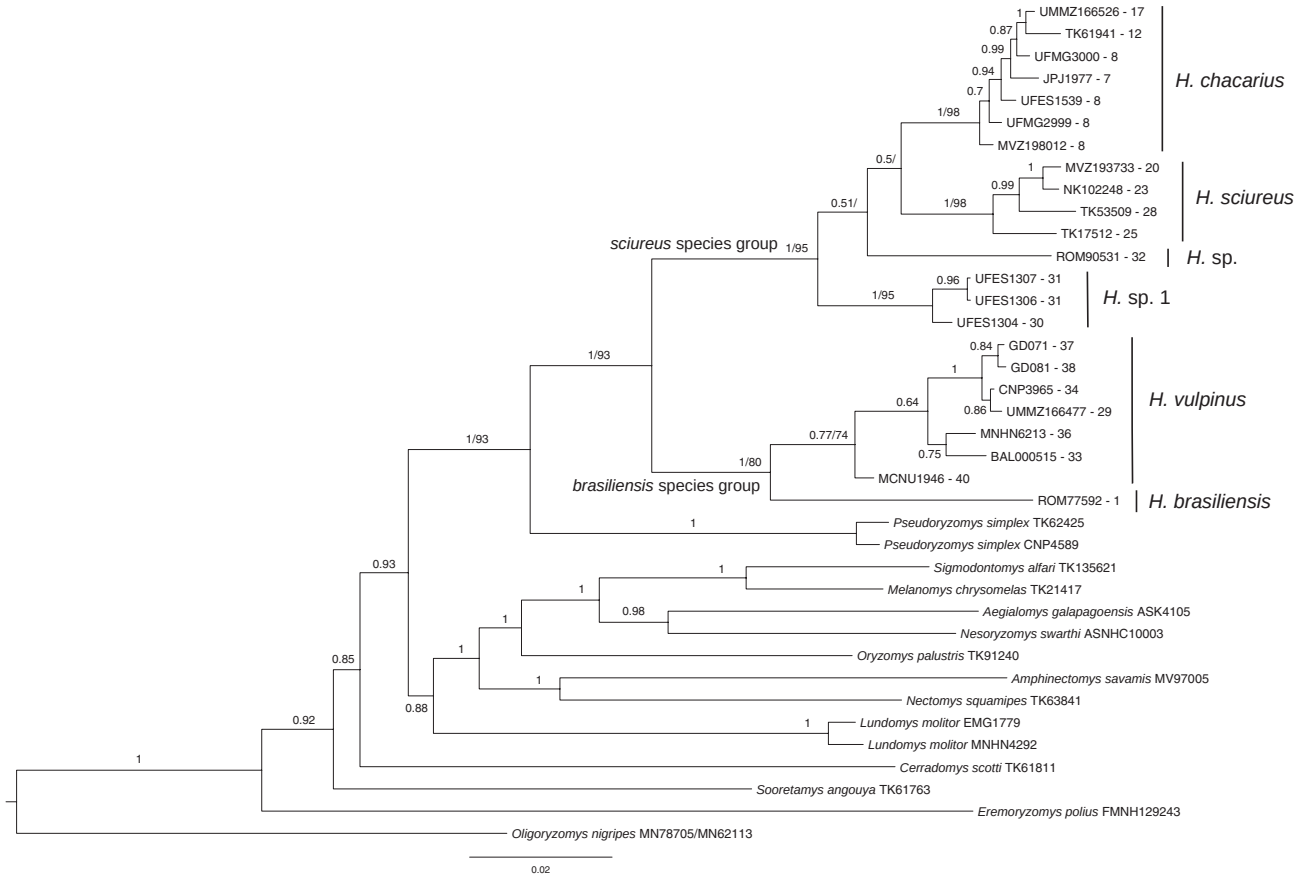

Fig. 4.—Genealogical relationship obtained in the Bayesian analysis of a concatenated matrix of 4 genes *Cytb*, *Rbp*3, *Adh*1-I2, and *Fgb*-I7 of the genus *Holochilus*.

The limits of Holochilus

The most unexpected result of our study is the lack of support for the monophyly of *Holochilus* found in the analysis of the *Cytb* matrix. *Cytb* variants of *Holochilus* fall into well-supported clades, the *brasiliensis* and *sciureus* species groups, which are not sister to each other (Fig. 2; Supporting Information S2). *Holochilus* is recovered as paraphyletic with respect to *Pseudoryzomys*. However, the paraphyly of *Holochilus* is not well supported given that the nodes involved lack significant support. In this regard, it is important to note that the 3 nuclear loci matrices analyzed recovered a monophyletic *Holochilus*. All 3 matrices had the same (*Adh*1-I2 and *Rbp*3) or comparable (*Fgb*-I7) outgroup selection to the *Cytb* matrix, and analyses of 2 of these matrices (*Adh*1-I2 and *Rbp*3) showed strong support for monophyly of *Holochilus* (Fig. 3; Supporting Information S3) with *Adh1*-I2 and *Fgb*-I7 doing so with high statistical support. Similarly, the monophyly of *Holochilus* is also recovered in the concatenated analyses (Fig. 4; Supporting Information S4). Still, it would be of interest to further evaluate the monophyly of *Holochilus* and species groups with a phylogenomic approach (see Lessa et al. 2014). If future studies reject the hypothesis of a monophyletic *Holochilus*, as presently understood, there is a generic name, *Holochilomys* Brandt, 1855, available to allocate to the *brasiliensis* species group. The history of this obscure name, which constitutes a good example of the complex taxonomic history of Neotropical rodents, is well narrated in Voss and Abramson (1999) and Gonçalves et al. (2015).

Regardless of the relationships between the 2 species groups of *Holochilus*, they are markedly distinct (Massoia 1976, 1981; Voss and Carleton 1993; Carleton and Olson 1999; Pardiñas and Galliari 1998; Pardiñas 2008; Gonçalves et al. 2015) and easily differentiated morphologically. Differences include fur length (short and close in the *sciureus* species groups versus dense and luxurious in the *brasiliensis* species group), tail length (shorter than the head and body versus about as long as the head and body or slightly longer), presence of hypothenar pad (occasionally present versus absent), number of mammae (8 or 10 versus always 8), size of the postorbital ridges (weakly to well expressed versus well expressed), length of the palate (usually short versus long), visibility of the subsquamosal fenestra (often occluded by an expanded hamular process or by an internal crest or septum of the periotic versus always distinct and patent), shape of the upper incisors (flattened laterally, with distinct labial bevel, lowers flattened laterally with lingual bevel versus somewhat flattened medially, with presumptive labial bevel, lowers unmodified), number of caudal vertebrae (29–32 caudal vertebrae in *chacarius* versus 33–35 in *vulpinus*), 2*n* (44–56 versus 35–40), and numerous traits regarding molar occlusal morphology (e.g., absence of mesoloph-like structures in *chacarius* versus its presence in *brasiliensis*). In this regard, it is of much interest to assess the phylogenetic

position of the recently described *H. lagigliai*, which displays a mixture of traits present in both the *sciureus* and *brasiliensis* groups (Pardiñas et al. 2013).

The brasiliensis species group

The distinction of *H. vulpinus* (including *darwini*) from *H. brasiliensis* has been disputed throughout the taxonomic history of the genus. Populations currently assigned to *H. vulpinus* (Gonçalves et al. 2015) have been classically considered to be part of *H. brasiliensis* either as representing the typical form (e.g., Musser and Carleton 2005; Pardiñas and Teta 2011) or as a subspecies (e.g., Massoia 1971; Freitas et al. 1983). Our results support the recognition of *H. vulpinus* at the species level, given that haplotypes from specimens assigned to this form fall in a strongly supported clade (PP = 1; BS = 100) that exhibited an average genetic distance of 0.089 at the *Cytb* gene from the clade of *H. brasiliensis* (a larger value than the 0.068 observed for the comparison of *H. chacarius and H. sciureus*), facts that are in accordance with the morphological differences summarized by Gonçalves et al. (2015).

Our sampling of *H. brasiliensis* is limited to 1 haplotype generated from a specimen collected approximately at Viçosa, 180 km SE from the type locality of this taxon (i.e., Lagoa Santa, Brazil, as restricted by Hershkovitz 1955; Fig. 1). Unfortunately, the skull of this specimen cannot be found (B. K. Lim, Royal Ontario Museum, pers. comm.) and as such we did not examine it. However, the assessment of 3 specimens (Museu de Zoologia, Departamento de Biologia Animal, Universidade Federal de Viçosa, Minas Gerais, Brazil; MZUFV 372, MZUFV 1935, and MZUFV 4068) collected at Viçosa allowed us to verify that in that area, in addition to *H. sciureus*, *H. brasiliensis* is also present. Given the paucity of available samples of *H. brasiliensis* and the ~ 1,600 km distance between representative samples of *H. brasiliensis* and *H. vulpinus*, it is clear that additional sampling and analyses are needed to clarify the geographic limits of both species, particularly in southern Brazil and Paraguay (Fig. 1). Such analyses are also needed to rule out the possibility that these lineages are not at opposite ends of an isolation-by-distance pattern of geographic structure. In this regard, it is necessary to evaluate if *H. brasiliensis*, as currently understood (i.e., Gonçalves et al. 2015), is a homogenous species or if geographic structure is present across its wide distribution.

H. vulpinus is distributed throughout a vast portion of the La Plata river Basin, including Paraguay, east-central Argentina, Uruguay, and the Brazilian state of Rio Grande do Sul (Massoia 1976, 1981; Marques 1988; Gonçalves et al. 2015); in the recent past, the species distribution was larger than present (Formoso et al. 2010; Pardiñas and Teta 2011). Gonçalves et al. (2015) consider *H. vulpinus* as monotypic, although Massoia (1976), based on morphometrics, treated *darwini*—with its type locality at the southern border of the range of the genus—as a distinct subspecies. Our results invite reevaluation of the subspecific scheme within *H. vulpinus*. Across its large distribution, *H. vulpinus* has a marked phylogeographic structure (Figs. 1 and 2); the mitochondrial analysis identified 2 allopatric main clades that on average diverge by 3.3% at the *Cytb* locus. A northern clade (PP = 1; BS = 100), which exhibits minimal genetic variation (0.2%), is distributed in Paraguay as well as the Argentinean provinces of Corrientes and Entre Ríos (localities: 29, 34, 37, 38, 42–44; Fig. 1B; Table 1). In contrast, the southern clade (PP = 0.88; BS = 63) is more variable (1.2%) and occurs in the province of Buenos Aires, Argentina, Uruguay, and in the state of Rio Grande do Sul in southern Brazil (localities: 33, 35, 36, 41; Fig. 1B; Table 1). Remarkably, these 2 clades are chromosomally different; the northern clade has a $2n = 48–56$ and the southern clade has $2n = 35–40$ (Riva et al. 1977; Freitas et al. 1983; Nachman 1992a). As such, increased geographic sampling is needed to include specimens from the type locality of *H. vulpinus* (the eastern margin of the Uruguay River in the state of Rio Grande do Sul between Itaqui and the Brazilian–Uruguayan border; Fig. 1—Cerqueira 1975). This sampling would help clarify if the name *vulpinus* is applicable to both clades or to only one. In turn, if *vulpinus* is restricted to the northern clade, the name *braziliensis* Wathersouse, 1839 (not *brasiliensis* as spelled in Gonçalves et al. 2015:335) may be used to the southern clade. Whereas, if *vulpinus* applies to the southern clade, *braziliensis* (and its proposed substitute name *darwini* Thomas, 1897) would be rendered as a junior synonym of *vulpinus*, thus no name would be available for the northern clade. We note that the name *multannus* Ameghino 1889—based on a fossil material from the northern Buenos Aires province and earlier regarded as junior synonym of *brasiliensis* (cf. Massoia and Pardiñas 1993)—is available and could be used for the northern clade if that group is shown to also be distributed in northern Buenos Aires. In any case, a detailed evaluation of the patterns of morphological and chromosomal variation, guided by the *Cytb* genealogy, is needed to establish which classification scheme best reflects the internal variation of *H. vulpinus* as currently understood.

The sciureus species group

Pardiñas and Galliari (1998) and Pardiñas and Teta (2011; see also Percequillo 2006) revealed morphological characters allowing a clear distinction between *H. chacarius* and *H. sciureus*. In particular, dental characteristics display consistent differences between both taxa, including lophid shape (with acute outer margins in *H. chacarius* versus with strongly acute outer margins and more prismatic in *H. sciureus*); the anteromedian fossetid (subcircular and large versus transversally elongated and small); and the metaflexid (scarcely developed, not reaching the midline of the tooth versus well developed, freely connected with the protoflexid in subadults). Our results based on the analysis of *Cytb* gene sequences corroborate the distinction of both species; *sciureus* and *chacarius* appear strongly supported (*chacarius*: PP = 1; BS = 99; *sciureus*: PP = 1; BS = 98) and diverge on average by 6.8 %.

Holochilus sciureus.—Our sampling lacks representatives of the *sciureus* species group from the Brazilian state of Minas Gerais, where the type locality of *H. sciureus* lies (Fig. 1; Hershkovitz 1955), and from most of the eastern part of the range of *H. sciureus* (cf. Brandão and Nascimento 2015; Gonçalves et al. 2015). Even so, for consistency with traditional usage, we here refer to the large clade found in Suriname;

western, central, and southern Brazil; and in portions of Peru, Bolivia, and Argentina as *H. sciureus*. Our sampling of *H. sciureus* includes 13 specimens collected over a large geographic area (Table 1; Fig. 1). The *Cytb* genealogy recovered for this species indicates geographic structure (Fig. 2) consisting of 4 highly supported allopatric clades, as follows: 1) 1 from northern Suriname (formed by 2 haplotypes from localities 25 and 26; PP = 0.96; BS = 82); 2) another from southern Suriname (locality 24; PP = 1; BS = 100); 3) a clade comprising Peruvian samples (localities 27 and 28 [specimen TTU76303 lacks specific locality data]; PP = 1; BS = 93); and 4) the last one composed of samples from Entre Ríos in Argentina, Santa Cruz in Bolivia, and Amazonas in Brazil (localities 19–23, 29; PP = 1; BS = 84). Relationships among these 4 clades are highly resolved (Fig. 2). Observed genetic divergence between pairs of clades of *H. sciureus* range from 2.0% for the comparison of clades 3 and 4 to 3.4% for the comparison of the Surinamese clades 1 and 2. Interestingly, the southern Suriname haplotype is genetically closer to Peruvian, Brazilian, and Bolivian haplotypes than it is to geographically much closer haplotypes from northern Suriname. This is a pattern also seen in *Oligoryzomys* (Hanson et al. 2011). Our results suggest that *H. sciureus* has a long history in its current broad distribution; future research is needed to clarify the drivers behind the observed within-species diversity and why the Guiana Shield area is more phylogeographically diverse than the vast area ranging from the Brazilian state of Amazonas, to central Bolivia and central Argentina.

Different taxa are currently under the synonymy of *H. sciureus* (Gonçalves et al. 2015), including *H. amazonicus* Osgood 1915, *H. guianae* Thomas 1901, *H. incarum* Thomas 1920, *H. nanus* Thomas 1901, and *H. sciureus berbicensis* Morrison-Scott 1937. Gonçalves et al. (2015) considered *H. sciureus* as being monotypic. A decade earlier, Barreto and García-Rangel (2005) recognized 2 subspecies, *H. s. berbicensis* and *H. s. sciureus* (with *venezuelae* in its synonomy, which is considered by Gonçalves et al. 2015, as a distinct species). Our sampling precludes unambiguously assigning any of the available names to the clades discussed above. Notwithstanding, our results suggest that as currently understood, even after removing the lineages here referred as *H*. sp. 1 and *H*. sp 2. (see below), *H. sciureus* may encompass more than 1 lineage that warrants subspecific (or specific) recognition. Again, a molecular-based analysis with a larger geographic coverage, accompanied with a comprehensive assessment of the pattern of morphological variation, is needed to gain an adequate picture of the variation of *H. sciureus*.

Future sampling should include representatives of the form *venezuelae*, as some authors (e.g., Aguilera and Pérez-Zapata 1989; Gonçalves et al. 2015) suggest this form represents a distinct species, whereas others consider it to be either a subspecies (e.g., Linares 1998) or a synonym of *H. sciureus* (e.g., Voss and Carleton 1993; Barreto and García-Rangel 2005). Interestingly, Venezuelan samples were found to be morphometrically different from Bolivian and Brazilian samples, suggesting they represent distinct lineages (Carleton and Olson 1999). Future sampling of molecular-based studies should include topotypical material as a way to tie names to clades with a good certainty.

Holochilus chacarius.—Massoia (1976) recognized 2 subspecies within *H. chacarius*, *H. c. chacarius* in the Paraguayan Chaco and northeastern Argentina south to Buenos Aires province and *H. c. balnearum* in southern Bolivia and northwestern Argentina, although Reig (1986) ranked the latter as a distinct species. Our sampling in the western range of the species is limited to 1 specimen (JPJ1977) from Jujuy province (northwestern Argentina; locality 7; Table 1; Fig. 1); this animal was collected approximately 350 km N of the type locality of *balnearum* and in the same piedmont sylvan environment (southern Yungas). The *Cytb* haplotype recovered from JPJ1977 is not sister to a clade form by western haplotypes, but nested within it. This result does not provide support to a distinction between *balnearum* and *chacarius* and is in agreement with the treatment given by Musser and Carleton (2005) who considered both taxa as synonyms. Clearly, this issue needs further analysis given that these taxa can be differentiated by karyotypes and measurements (Massoia 1976; Gonçalves et al. 2015).

It is of interest to note the low genetic variation of *H. chacarius Cytb* haplotypes (average within clade genetic divergence is 1.0%) and the lack of detected geographic structure. These observations are compatible with a scenario in which current populations of *H. chacarius* are relatively young and would have colonized their current distribution recently. A detailed phylogeographic study including more specimens and additional loci is needed to test this hypothesis.

Two additional species-level lineages.—In addition to providing further support for the distinction of *H. chacarius* and *H. sciureus*, our study provides evidence for the existence of 2 additional species-level lineages within the *sciureus* species group, which are herein referred as *H.* sp. 1 and *H*. sp. 2 (Fig. 2; Supporting Information S2). These 2 lineages were identified on the basis of the analysis of mitochondrial sequences of 2 and 1 individual(s), respectively. The sequence of the specimen of *H*. sp. 2 was obtained from a piece of dry skin and is shorter (577 bp) than the other analyzed sequences. The study of additional sequences from other specimens, together with an assessment of morphological and karyotypic variation, is needed to test the hypothesis that these specimens belong to 2 species level lineages distinct from those here recognized as *H. brasiliensis*, *H. chacarius*, *H. sciureus*, and *H. vulpinus*.

Holochilus sp. 1 is so far recorded from 2 localities in the central Brazilian state of Tocantins (Lagoa da Confusão and Parque Estadual do Cantão). Specimen UFES-MAM1304 from Parque Estadual do Cantão, for which no *Cytb* sequence is available, is tentatively referred to *H*. sp. 1 because its *Rbp*3 and *Fgb*-I7 sequences are sister to those gathered from specimens from Lagoa da Confusão. *H*. sp. 1 has diverged at the *Cytb* gene on average by 7.8% from *H. chacarius*, 7.9% from *H*. sp. 2, and 8.2% from *H. sciureus* (divergences with species of the *brasiliensis* species group are above 13%; Table 2). We note that the recording locality of *H*. sp. 1 is as close to the type locality of *H. sciureus* as that of any sample we allocate to *H. sciureus*. Therefore, until specimens from the eastern distributional range of the *sciureus* species group, particularly from Minas Gerais (where the type locality of *H. sciureus* lies; Fig. 1A),

are included in the analysis, we cannot rule out the possibility that *H*. sp. 1 represents in fact the true *H. sciureus* (or 1 of the taxa currently under its synonymy). As stated above, we use the name *H. sciureus* in a way to be consistent with traditional usage but acknowledge that until topotypical material is assessed, nomenclatorial uncertainty will remain.

Holochilus sp. 2 is represented by a single Colombian specimen (ROM90531) collected at Puerto López in the department of Meta (locality 32). *H*. sp. 2 has diverged at the *Cytb* gene by 6.7, 6.8, and 7.9 % from *H. chacarius*, *H. sciureus*, and *H*. sp. 1, respectively. Gardner and Patton (1976:41) karyotyped a specimen from Villavicencio, which is approximately 70 km W Puerto López, and reported a $2n = 50$, FN = 58 for it, which is not present in the recorded karyotypic variation of *H. venezuelae* and *H. sciureus* (see above). This result reinforces our suggestion that populations from central Colombia may represent a distinct species of *Holochilus*; we note that no name is available for it. However, Puerto López is not far (approximately 600 km SW) from El Yagual, the type locality of *H. venezuelae*, and both belong to the same fluvial basin, the Orinoco drainage. Beyond putative cytogenetics differences, in a genus that exhibits high levels of inter- and intraspecific chromosomal variation (see, for example, Nachman 1992a, 1992b), the possibility exists that our *H*. sp. 2 is in fact *H*. *venezuelae* and deserves future examination.

Biogeography

Holochilus is 1 of the most widely distributed genera of the sigmodontine radiation, inhabiting forested and open areas in a large fraction of lowland South America. As discussed above, species of *Holochilus* fall into 2 species groups, supporting the early morphological-based hypothesis advanced by Massoia (1976, 1980; see also Voss and Carleton 1993; Carleton and Olson 1999). The *brasiliensis* species group is distributed throughout the Atlantic coast of southeastern Brazil, central and northeastern Argentina, Paraguay, and Uruguay, whereas the *sciureus* species group is widely distributed in northern and central South America south to central Argentina. The groups are sympatric in central and northeastern Argentina, as well as eastern Paraguay. The fact that the La Plata basin encompasses more alpha and phylogenetic diversity than other areas of the continent may suggest a southern origin for the genus. The fossil record of these marsh rats is mostly restricted to remains from the Late Pleistocene and Holocene (Pardiñas and Teta 2011). The oldest occurrence of *Holochilus* sensu stricto (i.e., excluding *primigenius*) is from Middle Pleistocene deposits (approximately 1 Ma) in southeastern Buenos Aires province, Argentina (Pardiñas 2004); morphologically, this material is similar to representatives of the *brasiliensis* species group, implying that the split between species groups of *Holochilus* would have occurred before this time. Machado et al. (2014) infers an Early Pleistocene age for the stem group of *Holochilus*. However, a better resolution of species limits within the genus as well as a more robust phylogenetic species tree, which should include *H. lagigliai*, are needed before a detailed scenario of historical biogeography can be advanced for the genus.

An additional issue that deserves mention is the fact that some species of *Holochilus* present a contrast between levels of cytogenetic variation and of phylogeographic structure. *H. sciureus* shows across its large geographic range a conserved karyotype of $2n = 55–56$ (Gardner and Patton 1976; Vidal-Rioja et al. 1976; Baker et al. 1983; Freitas et al. 1983) but a marked phylogeographic structure. Whereas, the opposite pattern of large chromosomic variation ($2n = 48–56$—Vidal-Rioja et al. 1976; Nachman and Myers 1989; Nachman 1992a, 1992b) and lack of phylogeographic structure characterizes *H. chacarius*. The apparent inverse relationship between chromosomic variation and phylogeographic structure, and more important its biological meaning, if any, is unknown.

In conclusion, the geographic patterns of molecular variation presented constitute a preliminary foundation for a more detailed and comprehensive characterization of this ubiquitous genus of large semiaquatic sigmodontines, which is also of economic relevance. Future taxonomic studies should expand the geographic, taxonomic, and multilocus analyzed here, particularly in areas of Atlantic coastal Brazil as well as the Amazon and Orinoco basins, and include topotypical material as a way to unambiguously tie morphologically described taxonomic names with molecularly delineated clades.

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Supporting Information

The Supporting Information documents are linked to this manuscript and are available at Journal of Mammalogy online (jmammal.oxfordjournals.org). The materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supporting data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Supporting Information S1.—Details of the DNA sequences analyzed for each specimen included in the present study.

Supporting Information S2.—Results of the maximum likelihood phylogenetic analyses of 66 haplotypes of the *Cytb* gene of the genus *Holochilus*.

Supporting Information S3.—Results of the maximum likelihood phylogenetic analyses of DNA sequences of the loci *Rbp*3, *Adh*1-I2, and *Fgb*-I7 of the genus *Holochilus.*

Supporting Information S4.—Results of the maximum likelihood phylogenetic analyses of a concatenated matrix of four genes *Cytb*, *Rbp*3, *Adh*1-I2, and *Fgb*-I7 of the genus *Holochilus*.

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