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Species groups and the evolutionary diversification of tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae)

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We present the most comprehensive study to date of species groups in *Ctenomys* (tuco-tucos), a species-rich genus of Neotropical rodents. To explore phylogenetic relationships among 38 species and 12 undescribed forms we sequenced the complete mitochondrial cytochrome-*b* genes of 34 specimens and incorporated 50 previously published sequences. Parsimony, likelihood, and Bayesian phylogenetic analyses were performed using additional hystricognath rodents as outgroup taxa. The basal dichotomy of *Ctenomys* splits *C. sociabilis* from the remaining tuco-tucos, within which 8 main species groups were identified: *boliviensis*, *frater*, *mendocinus*, *opimus*, *magellanicus*, *talarum*, *torquatus*, and *tucumanus*. Whereas most of these groups refer to previous clades proposed on the basis of chromosomes or morphology, the *torquatus* and *magellanicus* species groups are novel taxonomic hypotheses. However, relationships among species groups are poorly resolved. Furthmore, the positions of *C. leucodon*, *C. maulinus*, and *C. tuconax* are conflicting or unresolved, and they might represent additional independent lineages. On the basis of molecular dating, we estimate that most species groups originated approximately 3 million years ago.

Key words: *Ctenomys*, cytochrome *b*, molecular dating, species groups, subterranean rodents, systematics, tuco-tucos

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The Neotropical region hosts a diverse, yet incompletely characterized, variety of mammals. During the last few decades field surveys, coupled with collection-based studies and molecular analyses, have helped characterize this complex mammalian fauna. Echimyids (spiny rats) and ctenomyids (tuco-tucos) are the 2 most diverse families of South American hystricognaths. *Ctenomys*, the sole living genus of the family Ctenomyidae, is poorly known because of the partial characterization of its alpha diversity and the unknown processes behind its diversification. Despite a moderate degree of morphological and ecological diversity, *Ctenomys* is characterized by high species richness (approximately 60 recognized living species—Woods and Kilpatrick 2005). Speciation is considered to be rapid for the genus to have reached its current diversity since its appearance in the late Pliocene (Reguero et al. 2007; Verzi et al. 2010).

Species of *Ctenomys* are distributed from southern Peru and southern Brazil to Tierra del Fuego through parts of Chile and most of Argentina, Bolivia, Paraguay, and Uruguay (Reig et al. 1990; Fig. 1). Tuco-tucos occur in a wide variety of habitats, from the Andean Puna above 4,000 m to the coastal dunes of the Atlantic, and from the mesic and humid Pampas to the dry Chaco and Monte desert. They have the largest known range of chromosomal variation of any mammal genus, with diploid numbers ranging from 2n = 10 to 2n = 70 (Cook et al. 1990; Novello and Lessa 1986). Reig (1989) suggested that tuco-tucos underwent a process of explosive radiation (see also Castillo et al., 2005; Cook and Lessa 1998). Efforts directed at revealing the macroevolutionary pattern of diversification of *Ctenomys* have been hampered by a lack of understanding of species limits and of phylogenetic relationships among them. For example, Sage et al. (1986) indicated that the genus was in a state of ''taxonomic chaos.'' Early efforts of establishing subgenera (Osgood 1946) or

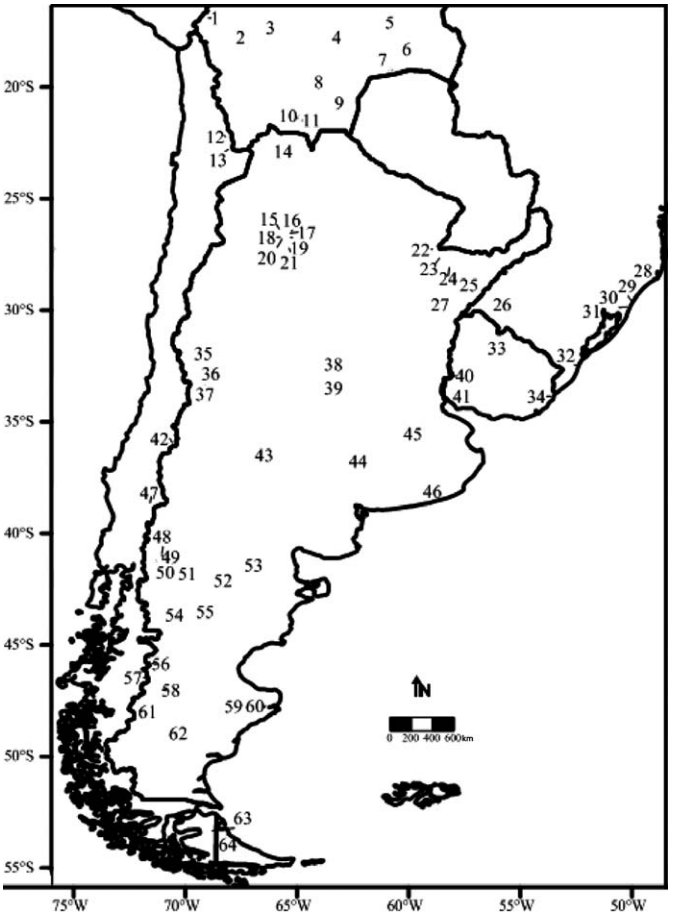


Fig. 1.—Map of collecting localities of *Ctenomys* used in the present study. Locality numbers refer to those of Appendix I.

sections (Ellerman 1940) for *Ctenomys* were later refuted (D'Elía et al. 1999). Other authors (Cabrera 1961) reduced many forms to synonyms or subspecies that were considered to be unfounded (Reig and Kiblisky 1969). In addition, many forms are known only from their original descriptions on the basis of one or a few specimens. Until the late 1960s classification of tuco-tucos was based primarily on pelage color, cranial morphology, and body size. More recent attempts were based on allozyme frequencies (Sage et al. 1986), karyotypes (Reig and Kiblisky 1969), penial morphology (Altuna and Lessa 1985; Balbontin et al. 1996), and sperm morphology (Feito and Gallardo 1982). Vitullo et al. (1988) proposed that different sperm variants appeared early in the radiation, possibly indicating a natural subdivision of the genus. D'Elía et al. (1999) noted that the group of species with asymmetric sperm is diphyletic in a phylogeny based on mitochondrial (mt)DNA sequences. Massarini et al. (1991) and Ortells and Barrantes (1994) delimited the *C. mendocinus* and Corrientes species groups, respectively, on the basis of chromosomal variation. Another proposal of classification was based on copy number of a major satellite DNA sequence (Rossi et al. 1993).

Taking into account the geographical distribution of species as an historical framework for the radiation, Contreras and Bidau (1999) proposed 8 species groups (Table 1); however,

Table 1.—Species groups of *Ctenomys* recognized in the present study compared with those suggested by Contreras and Bidau (1999).

Species	Contreras and Bidau (1999)	This study
boliviensis	Bolivian–Matogrossense	*boliviensis*
goodfellowi	Bolivian–Matogrossense	*boliviensis*
nattereri	Bolivian–Matogrossense	*boliviensis*
steinbachi	Bolivian–Matogrossense	*boliviensis*
conoveri	Bolivian–Paraguayan	*frater*
frater	Bolivian–Paraguayan	*frater*
lewisi	Bolivian–Paraguayan	*frater*
sp. Llathu	Not considered	*frater*
fodax	Patagonian	*magellanicus*
colburni	Patagonian	*magellanicus*
coyhaiquensis	Chilean spp.	*magellanicus*
haigi	Allied to *C. mendocinus*	*magellanicus*
magellanicus	Patagonian	*magellanicus*
sericeus	Patagonian	*magellanicus*
australis	*C. mendocinus* complex	*mendocinus*
flamarioni	Allied to *C. mendocinus*	*mendocinus*
mendocinus	Allied to *C. mendocinus*	*mendocinus*
porteousi	Allied to *C. mendocinus*	*mendocinus*
rionegrensis	Eastern	*mendocinus*
opimus	Chaco	*opimus*
fulvus	Not considered	*opimus*
saltarius	Not considered	*opimus*
scagliai	Chaco	*opimus*
pundti	Ancestral	*talarum*
talarum	Ancestral	*talarum*
minutus	Eastern	*torquatus*
lami	Not considered	*torquatus*
pearsoni	Allied to Corrientes	*torquatus*
perrensi	Corrientes	*torquatus*
roigi	Corrientes	*torquatus*
torquatus	Eastern	*torquatus*
argentinus	Chaco	*tucumanus*
latro	Chaco	*tucumanus*
ocultus	Chaco	*tucumanus*
tucumanus	Chaco	*tucumanus*
leucodon	Uncertain position	No species group
maulinus	Chilean spp.	No species group
sociabilis	Not considered	No species group
tuconax	Uncertain position	No species group

not all known species were considered. The first study with an explicit phylogenetic approach included only Argentinean and Bolivian species and was based on morphologic and karyotypic characters (Gardner 1990). Subsequently, Cook and Yates (1994) studied allozymic variation for Bolivian species, and Ortells (1995) examined the variation of karyotype G-band patterns in Argentinean species. More recently, Lessa and Cook (1998), D'Elía et al. (1999), Mascheretti et al. (2000), and Slamovits et al. (2001) analyzed sequences of the mitochondrial cytochrome-*b* gene of some Argentinian, Brazilian, Bolivian, Chilean, and Uruguayan species (Table 1). Most of these arrangements were given additional support by phylogenetic reconstructions on the basis of sequences of 2 nuclear introns (Castillo et al. 2005).

These studies during the last 2 decades have provided evidence for the identification of some species groups, whereas relationships among the groups remain poorly resolved. In addition, the taxonomic coverage of these studies has been limited to about one-half or less of the known species

of the genus. Similarly, geographic coverage is incomplete, with large geographic areas (e.g., Patagonia) remaining underrepresented. In molecular studies most species were represented by 1 specimen and very few came from type localities, which cast doubts on the application of several taxonomic names. Given this scenario, the present work is the most comprehensive taxonomic and geographic coverage in a phylogenetic analysis, with representatives of 38 species and 12 undetermined forms. Some species are represented by haplotypes gathered from more than 1 specimen, including from type localities. Our goals were to investigate species relationships and the timing of the main cladogenetic events within *Ctenomys*.

Materials and Methods

Taxonomic sampling.—The analysis was based on 71 complete sequences of the mitochondrial gene that encodes for the cytochrome-*b* protein. These sequences were from specimens of 38 nominal species (including 22 topotypes) and 12 undetermined forms. The outgroup consisted of 11 sequences from representatives of other families of Caviomorpha and of *Thryonomys* and *Bathyergus*, 2 African hystricognath genera.

Sequences of 34 specimens were newly acquired in the present study. The sequence of *C. sociabilis* was kindly provided by Ivanna Tomasco (Universidad de la República, Montevideo, Uruguay, pers. comm.). Details of the specimens studied are provided in Appendix 1 and Fig. 1. All parts of the study involving live animals followed guidelines of the American Society of Mammalogists (Gannon et al. 2007).

DNA extraction, amplification, and sequencing.—The complete mitochondrial cytochrome-*b* gene was amplified in two partially overlapping fragments obtained with primers MVZ05-tuco06 and tuco07-tuco14a (Smith and Patton 1999; Wlasiuk et al. 2003). Polymerase chain reaction (PCR) amplifications were carried out in a reaction volume of 40 μl containing 1.8 units of Taq polymerase (Biotools ByM Labs, Madrid, Spain), 20 μl of 3:100 total DNA dilution, 1.6 μl of each primer (10 μM), 1.6 μl of deoxynucleotide triphosphates (10 mM), and 4 μl of standard 10 mM buffer provided with the enzyme (including $MgCl_2$ 2 mM). The PCR amplification conditions were 3 min of initial denaturation, 31–35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 45–47°C, and 45 s of extension at 72°C followed by 10 min of final extension at 72°C. Negative controls were included in all PCR experiments. Amplicons were purified and sequenced at Macrogen Inc. (Seoul, South Korea) using the amplification primers. Partial sequences were assembled and edited with Sequence Navigator version 1.01 (Applied Biosystems, Inc., Foster City, California) and deposited in GenBank (accession numbers HM777474 to HM777506).

Phylogenetic analysis.—Alignment was done with MUSCLE (Edgar 2004) using 16 iterations and running in full mode with no manual adjustment required. Uncorrected genetic distances (p-distance) with pairwise deletion were computed for all pairs of sequences, and for within and between species, using MEGA 4.1 (Kumar et al. 2008).

Maximum parsimony (MP) analysis was done with TNT (Goloboff et al. 2008). The consensus was stabilized 10 times according to a factor of 100 collapses with tree bisection–reconnection (TBR). The relative support for each clade was obtained with 1,000 jackknife replicates (Farris et al. 1996), 33% character deletion, and searching 9 times for the best tree for each replicate. These results are shown as frequency differences (GC values, the difference in frequency between a group and the most contradictory group—Goloboff et al. 2003). Relative Bremer support (Bs—Goloboff and Farris 2001) values were obtained with TBR over the best trees found with TNT, taking into account relative amounts of favorable and contradictory evidence (0 = entirely unsupported, 1 = entirely uncontradicted).

Model-based analyses were done using the HKY85 model of sequence evolution (Hasegawa et al. 1985) selected among 56 nested models by ModelGenerator (Keane et al. 2006) through likelihood ratio tests using the Bayesian information criterion. Maximum-likelihood (ML) searches were carried out with PhyML (Guindon and Gascuel 2003) using 10 random starting trees optimized through subtree pruning and regrafting and nearest-neighbor interchange, 4 substitution categories with an estimated gamma distribution parameter (0.76), an estimated proportion of invariable sites (0.42), and the estimated transition/transversion ratio. Clade support was assessed by bootstrapping (B) with 100 replicates.

Bayesian inference was done with BEAST 1.5.2 (Drummond and Rambaut 2007) as a means to recover a phylogenetic hypothesis and simultaneously obtain an estimate of the divergence time for the main lineages of tuco-tucos. The same model of nucleotide substitution for ML was used with empirical base frequencies, 4 gamma categories, and partitioning into ''(1+2)+3'' (first and second codon positions in one partition and the third position in a separate partition). As indicated by previous analyses (not shown), the data are not clock-like; therefore, a relaxed uncorrelated lognormal clock was used together with no fixed mean substitution rate. This method incorporates the time-dependent nature of the evolutionary process without assuming a strict molecular clock. We used a Yule prior on branching rates because our analysis deals with a species-level phylogeny. Additionally, one prior was specified in the form of a calibration point as the time of the most recent common ancestor (tMRCA) for Caviomorpha (28.5–37 million years ago [mya]—Wyss et al. 1993). Four independent runs of 8 million generations were implemented, with the first 500,000 generations of each run discarded as burn-in. Posterior probabilities (P) were used as an estimate of branch support. The 95% highest posterior density intervals for the divergence time estimates were obtained for each node.

Results

Patterns and levels of variation.—Complete sequences (1,140 base pairs) of cytochrome *b* from 84 individuals (71 tuco-tucos and 13 other Hystricognathi) were analyzed and

Table 2.—Composition bias (%) and parsimony informative sites (PI) for the cytochrome *b* data set analyzed.

		Codon position		
Base	Total	1	2	3
T	30.5	27	41	24
C	25.8	21.6	25.2	30.7
A	31.4	30.5	20.3	43.3
G	12.3	20.8	13.7	2.5
PI	490.0	30.3	10.8	87.9

resulted in 597 (52.4%) variable sites, of which 490 were potentially parsimony informative and 68.2% of the variable changes were at third codon positions (Table 2). The estimated transition/transversion rate ratios is $k_1 = 3.19$ (purines) and $k_2 = 7.28$ (pyrimidines). The transition/transversion bias is $R = 2.256$.

The range of intraspecific divergence is 0.2–3.5% (*C. haigi* and *C. magellanicus*, respectively), whereas the average ($n = 14$) is 1.5%. Two species pairs are the most divergent (12.8%), *C. sociabilis–C. frater* and *C. sociabilis–C. leucodon*. *C. sociabilis* is the most divergent species (11.0% on average) from other tuco-tucos. The least divergent species pairs are comparable with those of some intraspecific comparisons. For example, *C. saltarius* and *C. scagliai*, and *C. coyhaiquensis* and *C. sericeus* differ by only 0.6% (see also Table 3).

Higher-level relationships.—Topologies obtained from the different methods are congruent, with the exception of discrepancies at weakly supported relationships within species groups. Therefore, only the ML topology is presented (lnL = −15458.521279), with the branch support values obtained from all three of the inference methods (Fig. 2). The parsimony analysis produced 12 shortest trees of 3,447 steps and a consistency index of 0.308 and a retention index of 0.568.

Ctenomys is monophyletic with strong support. The dichotomy at the base of *Ctenomys* splits into *C. sociabilis* and a clade of all other tuco-tucos, followed by subsequent divergence of *C. tuconax*, but these relationships are poorly supported. Within the remaining tuco-tuco clade, 8 relatively well-supported species groups are recovered (Fig. 2). The branching pattern and the relationships among these 8 clades and several other poorly supported clades are unstable.

The *opimus* group (A) is a relatively well-supported clade composed of the altiplano species (*C. opimus* and *C. fulvus*) and 2 species from the nearby Argentinean Chaco and the open highlands of Tucuman (*C. saltarius* and *C. scagliai*) that are sister to each other but with low sequence divergence (0.6%). *C. opimus* is recovered as paraphyletic relative to *C. fulvus* but poorly supported. The recovered divergence within the group is, on average, 3.94%, whereas *C. opimus* has the most divergent intraspecific haplotypes (1.8%). *C. maulinus,* a species of uncertain affinities in previous studies, is recovered as sister to the *opimus* group under MP and ML, although without strong support.

The *mendocinus* group (B), including *C. australis*, *C. mendocinus*, and *C. porteousi* (sensu Massarini et al. 1991), are closely allied with *C. flamarioni* and *C. rionegrensis*. However, the 3 haplotypes recovered from specimens assigned to *C. mendocinus* do not form a monophyletic group. One haplotype from Tupungato, an Andean locality in Mendoza, Argentina, is not sister to a clade formed by the other haplotypes of *C. mendocinus*, including one recovered from a specimen collected at the species type locality. The genetic divergences among members of the group are, on average, 2.4%.

The *talarum* group (C), which was referred to as the *C. pundti* complex by Tiranti et al. (2005), is formed by *C. pundti*

Table 3.—Divergence among pairs of species within species groups of *Ctenomys* for uncorrected p distance shown as percentage.

opimus group					
fulvus					
1.6	*opimus*				
5.4	5.5	*scagliai*			
5.7	5.8	0.6	*saltarius*		
mendocinus group					
porteousi					
1.2	*australis*				
1.7	1.9	*mendocinus*			
2.6	2.8	3.1	*flamarioni*		
2.5	2.9	2.8	3.4	*rionegrensis*	
talarum group					
pundti					
2.9	*talarum*				
torquatus group					
pearsoni					
2.3	*perrensi*				
3.1	1.9	*roigi*			
4.4	3.7	4.3	*torquatus*		
3.9	3.3	3.8	3.4	*lami*	
3.9	3.4	4.0	3.5	1.3	*minutus*
magellanicus group					
sericeus					
4.8	*colburni*				
3.5	5.4	*haigi*			
0.7	4.8	3.4	*coyhaiquensis*		
1.4	5.6	4.3	1.2	*fodax*	
5.0	0.5	5.6	5.0	5.7	*magellanicus*
tucumanus group					
latro					
2.0	*occultus*				
1.5	1.5	*argentinus*			
6.9	7.1	6.8	*tucumanus*		
boliviensis group					
nattereri					
2.2	*robo*				
5.4	5.7	*boliviensis*			
5.4	5.6	1.2	*goodfellowi*		
6.9	6.5	6.2	6.6	*steinbachi*	
frater group					
lewisi					
5.2	*frater*				
8.2	9.1	*conoveri*			
8.3	8.9	6.8	*C.* sp. (from Llathu)		

and *C. talarum* (node C) in a well-supported monophyletic clade. However, *C. talarum* is paraphyletic in relation to *C. pundti*. The intraspecific divergence among *C. talarum* ranges from 0.4% to 2.1%. The *C. talarum* group is the sister taxon to the *C. mendocinus* group.

The *torquatus* group (D) has *C. torquatus* (from Brazil and Uruguay) as a poorly supported sister to the remaining species of the group, which are grouped into 2 clades. One of them is well supported and is formed by *C. perrensi*, *C. roigi* (Argentina), and *C. pearsoni* (Uruguay). However, the 3 haplotypes of *C. perrensi* are not reciprocally monophyletic. The other clade is poorly supported and formed by 2 Brazilian species, *C. lami* and *C. minutus*. *C. minutus* is paraphyletic in relation to *C. lami*, and specimens of *C. minutus* from Osorio and *C. lami* from Chico Loma share a haplotype. Observed divergence within the *torquatus* group was, on average, 2.0%.

The *magellanicus* group (E) is well supported and composed entirely of Patagonian–Fueguian species (*C. coyhaiquensis*, *C. colburni*, *C. fodax*, *C. haigi*, *C. magellanicus*, and *C. sericeus*). The other Patagonian–Fueguian species (*C. maulinus* and *C. sociabilis*) are in divergent lineages. The haplotype from a specimen collected at the type locality (El Maiten, Chubut, Argentina) of *C. haigi* diverges substantially (>3.5%) from a clade that is composed of species assigned to *C. haigi* and samples from 2 additional localities in the northern Patagonian steppe (Somuncura and Talagapa, Chubut, Argentina). Haplotypes recovered from specimens of an undescribed form collected at Pichiñan and Quichaura (Chubut, Argentina) form a well-supported clade that is sister to the clade composed of *C. fodax*, *C. coyhaiquensis*, and *C. sericeus*. A haplotype from the type locality of *C. colburni* was closely related to the Fueguian *C. magellanicus*, and their divergence is minimal (0.5%). The recovered divergence within the group was, on average, 3.5%.

The *tucumanus* group (F) is relatively well supported and formed by species of northern distribution, including *C. argentinus*, *C. occultus*, *C. latro*, and *C. tucumanus* as successively basal lineages. The most divergent species is *C. tucumanus* (6.9%). Within the group, observed divergence was, on average, 4.3%.

The *boliviensis* group (G) is a well-supported monophyletic clade that includes species identified as the ''Boliviano–Matogrossense'' group (*C. boliviensis*, *C. goodfellowi*, and *C. nattereri*) by Contreras and Bidau (1999), and *C.* sp. from Robore, Bolivia (Lessa and Cook 1998), with interspecific genetic divergence averaging 4.8%. *C. steinbachi* is a poorly supported sister to this group. In addition, 3 undetermined Bolivian forms (''ita,'' ''monte,'' and ''minut'') form a clade that might be sister to the *boliviensis* group.

The *frater* group (H) is well supported and includes a Chaco and intermediate Andean elevation species, which was referred to as the Bolivian–Paraguayan group by Contreras and Bidau (1999). It includes *C. frater*, *C. lewisi*, *C. conoveri*, and *C.* sp. (from Llathu) and is sister to the clade formed by the other species groups and *C. leucodon*. The *frater* group has the highest observed divergence among species (up to 7.7%), with an average of 3.94%. The highest genetic distance among groups was found between species of the *C. frater* and the *C. tucumanus* groups (11.3%), with an average distance of 7.6%.

Estimates of divergence dates have broad confidence intervals (Fig. 3, Table 4). The tMRCA of *Ctenomys* was dated at 9.22 (6.4–12.6) mya. Most of the tMRCA for species groups were dated around 3 mya, and species groups from the eastern distribution of the genus were of more recent origin.

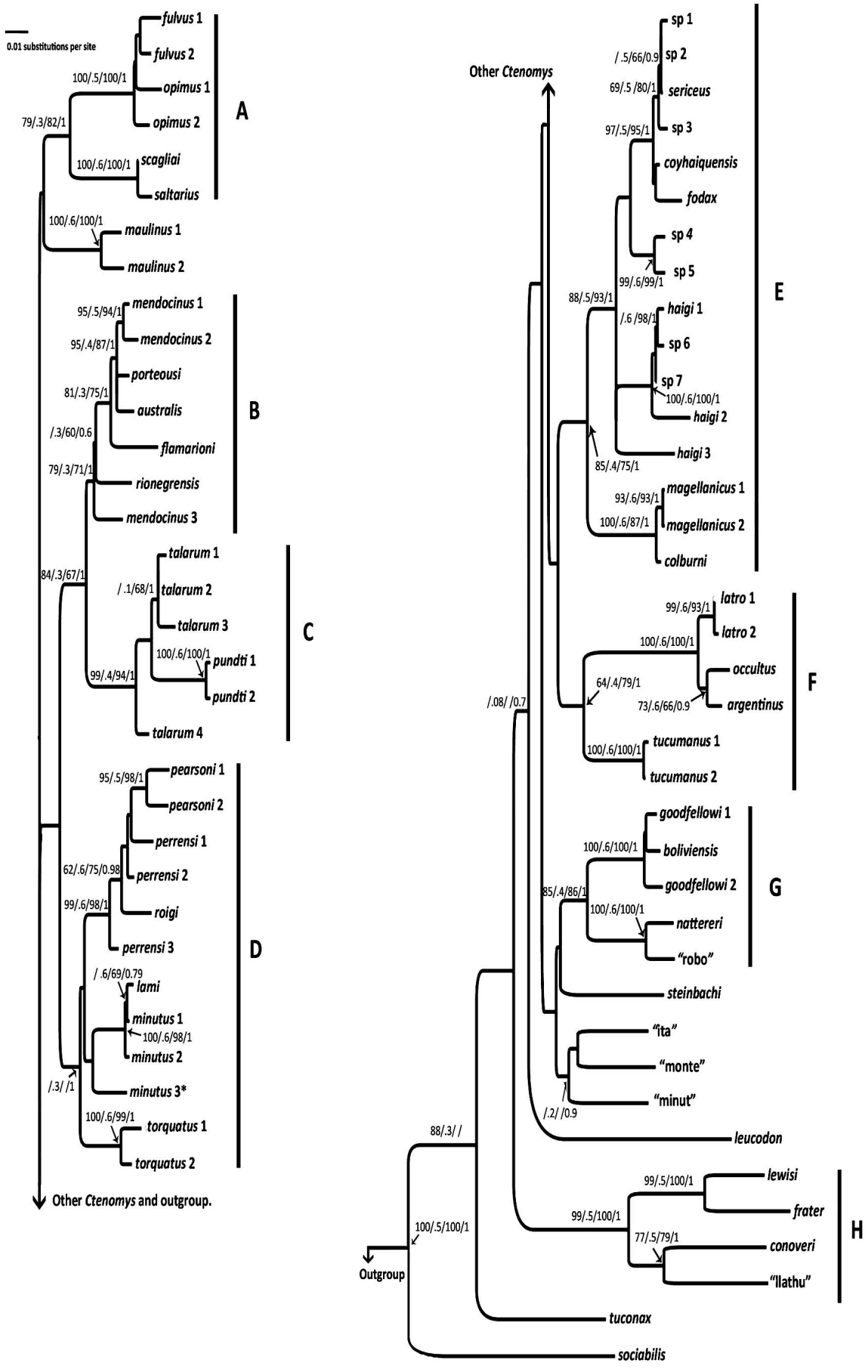
Discussion

Ctenomys is the most diverse genus of hystricognath rodents, but our current understanding of this diversity, both in terms of its alpha taxonomy and phylogenetic relationships, is inadequate. The present study, based on mtDNA sequences, has the broadest taxonomic and geographic coverage to date. We focus mainly on relationships among species, although some comments on species limits also are discussed.

Previous molecular phylogenetic studies (Castillo et al. 2005; Cook and Lessa 1998 D'Elía et al. 1999; Lessa and Cook 1998) found a polytomy at the base of the tuco-tucos clade, possibly reflecting the rapid, early diversification of the genus or saturation and loss of phylogenetic signal in the cytochrome-*b* gene. In our study the node at the base of *Ctenomys* leads to 2 lineages, *C. sociabilis* and a clade forming the remaining tuco-tucos. The sequence provided by Lara et al. (1996), and subsequently used by Lessa and Cook (1998), does not correspond to *C. sociabilis*, but probably to *C. haigi*. The identification of *C. sociabilis*, a social species whose behavioral ecology has been studied intensively (Lacey et al. 1997; Lacey and Wieczorek 2003), as sister to the remaining species of the genus was well supported by only MP bootstrapping. Therefore, this topology should be tested further by the analysis of nuclear DNA sequences due to the direct implications it has toward the understanding of the biogeographic history of the genus. For example, *C. sociabilis* is an austral species inhabiting the Argentinean province of Neuqeuen in northern Patagonia, and the oldest known fossil record assigned to *Ctenomys*, *C. uquiensis*, comes from Late Pliocene sediments of the northwestern Argentinean province of Jujuy (Verzi et al. 2010). This information needs to be integrated into a historical biogeographic hypothesis for *Ctenomys*.

The phylogeny has most species of *Ctenomys* in 8 relatively well-supported species groups. We did not associate names on the basis of their geographic distribution as done by Contreras and Bidau (1999). Instead, we refer to them using the names of the oldest species in each group. Eight main species groups were identified: *boliviensis*, *frater*, *mendocinus*, *opimus*, *talarum*, *magellanicus*, *torquatus*, and *tucumanus*. The *magellanicus* and *torquatus* groups are newly proposed in this study, although the *torquatus* group was not well supported. The other species groups were previously indentified, with some modifications, in earlier analyses.

Relationships among species groups are poorly supported, with the exception of the *mendocinus* and *talarum* groups,



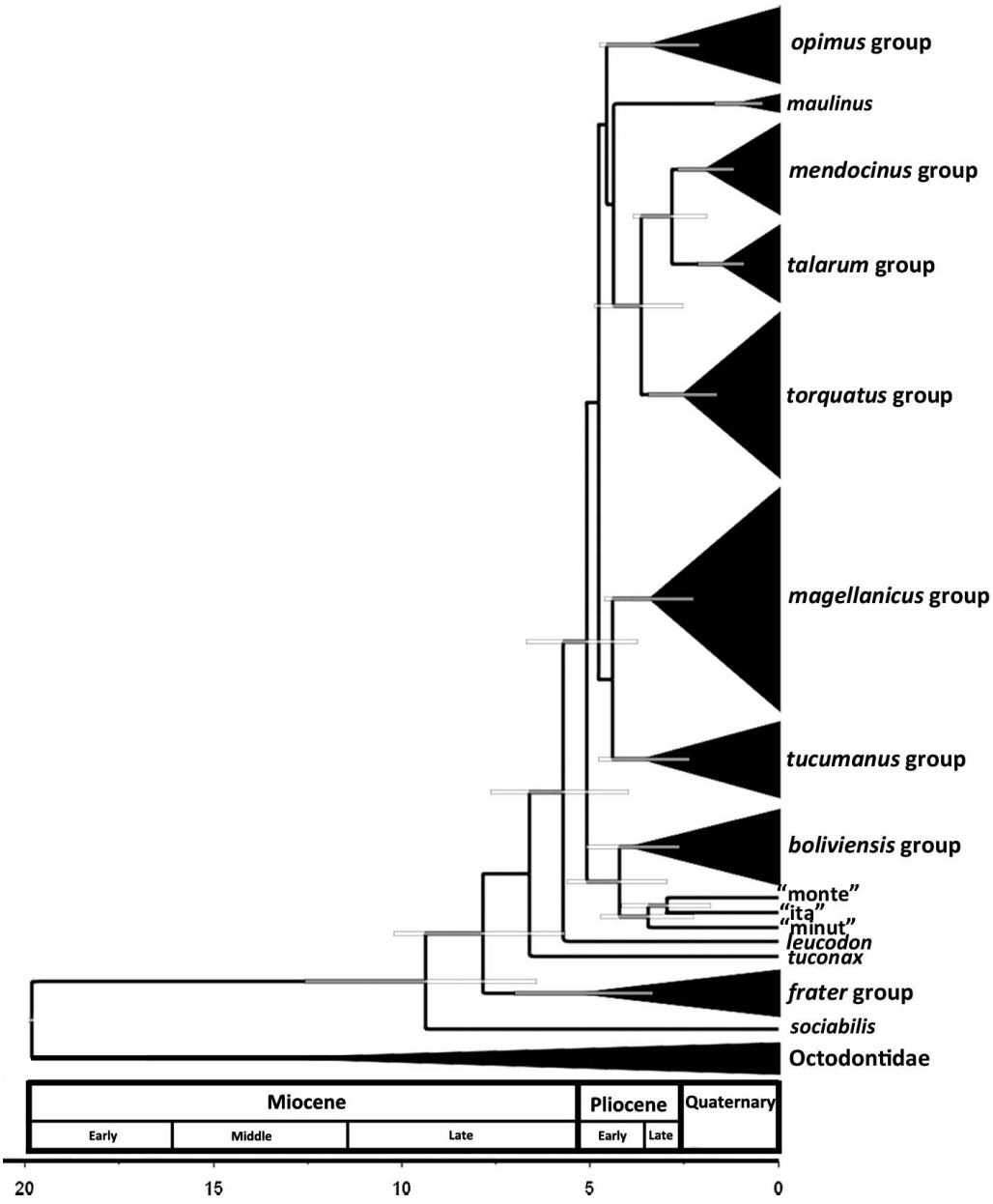


Fig. 3.—Bayesian tree with divergence dates from the relaxed uncorrelated lognormal clock analysis. The bars represent the 95% highest posterior density (HPD) interval for the divergence time estimates. Numbers indicate million years before present.

which are sister. The *torquatus* group is a poorly supported sister to this clade, and these 3 species groups comprise the eastern distribution of the genus. More effort is needed, however, to clarify the position of the undetermined forms referred to as ita, monte, and minut, which are recovered as a poorly supported clade, phylogenetically close to the *boliviensis* group.

The close relationship of *C. mendocinus*, *C. porteousi*, *C. australis*, and *C. azarae* (not included here), originally referred to as the *mendocinus* group, has been noted since their karyotypes were first described (Massarini et al. 1991). Subsequent explicit phylogenetic efforts (D'Elía et al. 1999) corroborated the grouping of these species and suggested that *C. flamarioni* and *C. rionegrensis* are also part of it. Our

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Fig. 2. The phylogenetic tree resulting from maximum-likelihood analysis of cytochrome-*b* gene sequences recovered from 71 specimens of *Ctenomys*. Haplotype labels follow specimen labels, as presented in Appendix I. Letters designate species groups as referred to in the text. Numbers indicate support from parsimony jackknife (%), relative Bremer support, likelihood bootstrap (%), and posterior probability (J/Bs/B/P). A node without numbers implies that the node has <60% of J and B and/or <0.7 P. * is a shared haplotype with *C. lami* from Chico Loma.

Table 4.—Estimates of divergence times recovered as mean estimates between different caviomorph lineages. Each value represents the estimated divergence time (mya) and 95% confidence interval. As in the text, *Ctenomys* species groups are referred to by the specific term.

Lineage	Divergence time
tMRCA Caviomorpha	32.03 (28.6–37.2)
tMRCA Cavioidea	21.89 (14.6–29.5)
(*Ctenomys*+Octodontidae/ Echimyidae) + Erethizontoidea	28.98 (23.4–34.5)
Ctenomys+Octodontidae/Echimyidae	23.18 (18.2–28.6)
Ctenomys+Octodontidae	17.97 (13.5–23.0)
tMRCA Octodontidae	12.34 (8.4–16.7)
tMRCA *Ctenomys*	9.22 (6.4–12.6)
All *Ctenomys* minus *sociabilis*	7.74 (5.7–10.2)
frater	4.97 (3.3–7.0)
All spp. groups minus *frater*	5.02 (3.7–6.7)
boliviensis	3.77 (2.6–5.1)
tucumanus	3.44 (2.4–4.8)
magellanicus	3.30 (2.6–4.6)
opimus	3.29 (2.1–4.8)
torquatus	2.46 (1.6–3.4)
talarum	1.44 (0.9–2.1)
mendocinus	1.85 (1.2–2.7)
talarum/mendocinus	2.78 (1.9–3.9)

analysis corroborates this grouping, which inhabits lowlands and mountainous regions across different ecoregions in the center of the distributional range of the genus. As noted by Massarini et al. (1991), the *mendocinus* group has a conservative diploid number (2n = 47–48) and morphology for the genus. However, the limited genetic divergence that is found among *C. australis*, *C. porteousi*, and *C. mendocinus* needs further taxonomic assessment. In addition, the phylogenetic position of the haplotype recovered from a specimen collected at Tupungato and currently assigned to *C. mendocinus* indicates that it might represent a species distinct from *C. mendocinus*. Furthermore, some nominal forms (e.g., *C. pontifex* and *C. coludo*) that might belong to this group have not been included in any phylogenetic study.

Ctenomys fulvus and *C. opimus* might be considered as conspecific because of their high sequence (Lessa and Cook 1998) and chromosomal (Gallardo 1991) similarity. We expand the *opimus* group to include *C. saltarius* and *C. scagliai*, which has only 0.6% sequence divergence. In the case of the ML and MP trees, *C. maulinus* is a poorly supported sister species to the *opimus* group, whereas in the Bayesian tree it is sister to the clade formed by the *C. mendocinus*, *C. talarum*, and *C. torquatus* groups.

Mascheretti et al. (2000) identified a group of Chacoan species formed by *C. argentinus*, *C. latro*, *C. occultus*, and *C. pilarensis*, which with *C. tucumanus* form our *tucumanus* group. *C. opimus* and *C. scagliai* were grouped under the Chaco group by Contreras and Bidau (1999), but these 2 species are recovered in our study as part of the *opimus* group. To investigate further the relationships of the species from northern Argentina a broader phylogeographic analysis including more representatives is needed.

Ctenomys talarum and *C. pundti* form a species group, which Tiranti et al. (2005) identified as the *C. pundti* complex. However, we refer to it as the *talarum* group because this is an older name than *pundti*. In addition, haplotypes of *C. talarum* form a paraphyletic group with respect to those of *C. pundti*, casting doubts on the taxonomic status of both taxa. As is the case with most taxa of *Ctenomys*, species limits within this group need to be evaluated further with the integration of more specimens, morphological characters, and nuclear DNA sequences.

The topology recovered within the *C. torquatus* group indicates the need for further assessment of the taxonomic status of *C. perrensi*, *C. pearsoni*, and *C. roigi*. Each of these nominal species has impressive chromosomal variation (Ortells and Barrantes 1994), but some of these species are not monophyletic. Similarly, pairwise distances between *C. minutus* and *C. lami* (0.4–2.7%) indicate low divergence among some haplotypes, and an analysis of cranial morphology did not find any substantial difference between them (Freitas 2005). Although the *torquatus* group lacks strong support, it was recovered by all methods. In addition, previous studies (Freitas 2005; Lessa and Langguth 1983) have commented on the morphological similarities among members of this group.

The *magellanicus* group comprises species from the Patagonian–Fueguian open areas and represents the only extant group to range into the southern end of South America. All individuals collected on the mainland south of the Senger River (~54° S), including those labeled as sp. 1, sp. 2, sp. 3, *C. sericeus*, *C. coyhaiquensis*, and *C. fodax*, might represent a single biological species, given the low observed genetic distances among the haplotypes examined. Similarly, the genetic distance between the haplotypes of *C. colburni* and *C. magellanicus* is minimal. In addition, *C. haigi* was not recovered as monophyletic, and the specimen labeled as *haigi* 3 is a topotype of *C. haigi*. The specimens labeled as *haigi* 1, *haigi* 2, sp. 6, and sp. 7 form a subclade within the *magellanicus* group. Whether the entire subclade represents 1 or more species is unclear. Several named forms of Patagonian taxa not included here (e.g., *C. emilianus*, *C. magellanicus osgoodi*) would have to be considered in future analyses.

Asymmetric sperm morphotype is found in southern species (*mendocinus* group, *magellanicus* group, *C. maulinus*, and *C. sociabilis*), which do not form a monophyletic group. This supports the suggestion of D'Elía et al. (1999) and Slamovits et al. (2001) that the asymmetric morph appeared more than once in the evolutionary history of *Ctenomys*. The uncertain position of *C. maulinus*, a species with the asymmetric sperm, and the lack of sequence data for *C. yolandae*, which has a third sperm morphology (Vitullo et al. 1988), hinders the recovery of the evolutionary history of this variable trait. Similarly, additional work is needed to understand the chromosomal evolution of the genus and the general pattern of diversification.

Divergence time estimates for the splitting of the lineages leading to the families Ctenomyidae and Octodontidae and

that of the tMRCA of Octodontidae (estimated around 17.97 and 12.34 mya, respectively) are older than previous estimates on the basis of 12S rRNA and growth hormone receptor sequences (Opazo 2005). Similarly, the tMRCA of *Ctenomys* was estimated at 9.22 mya, a value much older than previous estimates (3.7 mya—Castillo et al. 2005) or the evidence from the fossil record, which suggests that the split between Ctenomyidae and Octodontidae occurred not more than 9 mya (Verzi 2002). It should be taken into account that our analysis differs from previous ones on methods and sampling. More recently, Verzi et al. (2010) reported and described the oldest known tuco-tuco species, implying a minimum age for the genus of about 3.5 million years. Verzi et al. (2010) considered that the uncertainty of the age of *Praectenomys* (which is regarded as sister to *Ctenomys*) hampers a more definitive estimation of a maximum age for *Ctenomys*. Our estimations are based on a single locus, with large confidence intervals associated with them (Table 4).

The northern species groups occurring in Argentina and Bolivia (*boliviensis*, *frater*, *tucumanus*, and *opimus*) and the southern *magellanicus* group seem to be older (~3.5 million years) than those inhabiting central Argentina, eastern Brazil, and Uruguay, such as the *talarum* and *torquatus* groups that diverged around 2.0 mya. The inclusion of taxa missing in our analysis could change this date, and these estimates refer only to living members of species groups.

Our results are similar to those found by Cook and Lessa (1998) in identifying an increase in the diversification rate at the base of the tuco-tucos clade. After the basal split our results suggested an increase in diversification ~3 mya after some main lineages already had diverged. The cause of this diversification pulse remains obscure. The inclusion of more representatives of Caviomorpha and more calibration points and loci would provide a better understanding of the timing of the radiation.

Some nominal species appear to be polyphyletic or bear little or no divergence from other named species; however, we are not proposing formally any taxonomic change because our analysis is based solely on 1 gene and lacks specimens from several type localities. We concur with Freitas (2005) for the need for further integration of molecular and morphological analyses, including the study of type and topotype specimens to propose a classification scheme that better reflects phylogeny. Until these issues are addressed the distinctiveness of several taxonomic forms and the species diversity will remain elusive.

The lack of monophyly for several forms could reflect a disagreement between the inferred gene tree and the species tree because of random fixation of alternative ancestral haplotypes via incomplete lineage sorting (Neigel and Avise 1986). In addition, potential discrepancies could be caused by mtDNA introgression (Patton and Smith 1994). Despite these limitations, species groups could be identified using mtDNA sequences, and these were similar to groups identified by nuclear intron data (Castillo et al. 2005), morphology, or chromosomes (Contreras and Bidau 1999). However, a need exists for multilocus analyses to build on the previous studies by Castillo et al. (2005) and Galewski et al. (2005) that used loci in the closely related Echimyidae, including nuclear RAG-1 (Patterson and Velazco 2008), for a better understanding of phylogenetic relationships within *Ctenomys*.

Resumen

Presentamos aquí el estudio más exhaustivo hasta la fecha de los grupos de especies en *Ctenomys* (tuco-tucos), un género de roedores Neotropicales conocido por su riqueza específica. Para explorar las relaciones filogenéticas de 38 especies y 12 formas indeterminadas, se secuenció el citocromo b completo de 34 especímenes y se incorporaron 50 secuencias previamente publicadas. Se tuvieron en cuenta análisis por Parsimonia, Verosimilitud y Bayesianos empleando histrocognatos como grupo externo. La dicotomía más basal lleva a *C. sociabilis* por una parte y al resto de los tuco-tucos por otra. Dentro de los tuco-tucos, se identifican 8 grupos de especies: *boliviensis*, *frater*, *mendocinus*, *opimus*, *talarum*, *magellanicus*, *torquatus* y *tucumanus*. Mientras que la mayoría de los grupos aluden a clados identificados mediante estudios de cromosomas o morfología el grupo *torquatus* y *magellanicus* son hipótesis taxonómicas nuevas. Las relaciones basales entre los grupos de especies se encuentran con poco apoyo. La posición de *C. leucodon*, *C. maulinus* y *C. tuconax* son conflictivas o irresueltas y estas podrían representar linajes independientes. Adicionalmente, de acuerdo a nuestros estimados, los grupos de especies se habrían originado hace alrededor de 3 millones de años.

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APPENDIX I

List of the specimens of *Ctenomys* used in the present study. Accession numbers are indicated for those specimens whose sequences were retrieved from GenBank. See Fig. 1 for locality numbers and locations of sites. Museum and collection acronyms and personal field numbers are as follows: Argentina, Universidad de Mar del Plata (Matías Mora, IF); Instituto Argentino de Investigaciones de Zonas Áridas, Mendoza (Agustina Ojeda, AO); Centro Nacional Patagónico, Puerto Madryn (Proyecto National Geographic, PNG and Proyecto Localidades Típicas–Ulyses Pardiñas, LTU); Universidad Nacional de Patagonia, Esquel (Sergio Vincón, SV); Brazil, Instituo Oswaldo Cruz, Río de Janeiro (Claudio J. Bidau, CB); Universidad Federal Río Grande do Sul, Porto Alegre (Thales R.O. Freitas, TJ, CML and TR); and USA, Museum of Vertebrate Zoology, University California, Berkeley (Eileen Lacey, EAL).

Voucher numbers for new sequences are given after the accession number in parentheses.

Ctenomys argentinus.—Colonia Benitez, Argentina, 22 (AF370680).

Ctenomys australis.—Necochea, Argentina, 46 (AF370697, topotype).

Ctenomys boliviensis.—Las Lomitas, Bolivia, 4 (AF007038, topotype).

Ctenomys colburni.—Río Ecker, 47°07′S, 70°51′W, Santa Cruz, Argentina, 58 (HM777474/LTU191, topotype).

Ctenomys conoveri.—Carandayti, Bolivia, 9 (AF007055).

Ctenomys coyhaiquensis.—Chile Chico, Chile, 57 (AF119112, topotype).

Ctenomys flamarioni.—Taim, Brazil 32 (AF119107, topotype).

Ctenomys fodax.—Lago Blanco, 45°55′S, 71°18′W, Chubut, Argentina, 56 (HM777475/sv52, topotype).

Ctenomys frater.—Rancho Tambo Bolivia, 11 (AF007045).

*Ctenomys fulvus.—fulvus*1, San Pedro de Atacama, Chile, 13 (A0F370688, topotype); *fulvus*2, Turi, Chile, 12 (AF370687).

*Ctenomys goodfellowi.—goodfellowi*1, *goodfellowi*2, San Ramon Bolivia, 7 (AF007050-51).

*Ctenomys haigi.—haigi*1, Nahuel Huapi, Argentina 49 (AF422920); *haigi*2, Bariloche, Argentina 50 (AF007063); *haigi*3 Maiten, 42°3′S, 71°10′W Chubut, Argentina 51 (HM777476/sv62, topotype).

Ctenomys lami.—Beco dos Cegos, 30°51′S, 51°10′W, Rio Grande do Sul, Brazil, 31 (HM777477/TJ186, topotype).

*Ctenomys latro.—latro*1, *latro*2, Tapia, 26°36′S, 65°15′W, Tucumán, Argentina, 17 (HM777478/CB286 or C-04679, AF370704, topotype).

Ctenomys leucodon.—San Andres de Machaca, Bolivia, 1 (AF007056, topotype).

Ctenomys lewisi.—Iscayachi, Bolivia, 10 (AF007049).

*Ctenomys magellanicus.—magellanicus*1, Estancia Sara, 56°26′S, 68°11′W, Tierra del Fuego, Argentina, 64 (HM777479/PNG365); *magellanicus*2, Tres Arroyos, Tierra del Fuego, Chile, 63 (AF370690).

*Ctenomys maulinus.—maulinus*1, Pelechue, Chile 42 (AF370703); *maulinus*2, Rio Colorado, Chile 47 (AF370702).

Ctenomys mendocinus.—*mendocinus*1, Cerro de la Gloria, 32°52′S, 68°48′W, Mendoza, Argentina 36 (HM777480/AO59, topotype); *mendocinus*2, Las Heras, Argentina 35 (AF007062); *mendocinus*3, Tupungato, Argentina 37 (AF370695).

Ctenomys minutus.—*minutus*1, Praia do Barco 29°40′S, 48°58′W, Brazil, 29 (HM777481/CML431); *minutus*2, Jaguaruna 28°37′S, 49°01′W Rio Grande do Sul, Brazil, 28 (HM777482/TR40); *minutus*3 Osorio, 29°52′S, 50°12′W, Rio Grande do Sul, Brazil 30 (HM777483/TR02).

Ctenomys nattereri.—Santa Cruz de la Sierra, 17°36′S, 63°04′, Santa Cruz, Bolivia 4 (HM777484/CB3968 or C-03968).

Ctenomys occultus.—Simoca, 27°15′S, 65°21′W, Tucumán, Argentina 21 (HM777485/CB291 or C-04685).

Ctenomys opimus.—*opimus*1, Huancaroma Bolivia 2 (AF007042); *opimus*2, Tres Cruces Argentina, 14 (AF370700).

Ctenomys pearsoni.—*pearsoni*1 El Potrerillo Uruguay 34 (AF119108); *pearsoni*2 Limetas, 34°11′S, 58°6′, Colonia, Uruguay 41 (HM777486/EV1454, topotype).

Ctenomys perrensi.—*perrensi*1, Chavarría, 28°57′S, 58°34′W, Corrientes, Argentina 27 (HM777487/CB349 or C-04822); *perrensi*2, San Miguel, 27°59′S, 57°35′W, Corrientes, Argentina 25 (HM777488/CB554 or C-05503); *perrensi*3, Mburucuyá, 28°02′S, 58°13′W, Corrientes, Argentina 24 (HM777489/CB778 or C-05142).

Ctenomys porteousi.—Bonifacio 36°48′S, 62°13′W, Buenos Aires, Argentina 44 (AF370682, topotype).

Ctenomys pundti.—*pundti*1, Puente Olmos, 32°28′S, 63°19′W, Córdoba, Argentina 39 (HM777490/CB589 or C-03755); *pundti*2 Manantiales, 29°52′S, 63°54′W, Córdoba, Argentina 38 (HM777491/CB592 or C-04042).

Ctenomys rionegrensis.—Las Cañas, Uruguay 40 (AF119103, topotype).

Ctenomys roigi.—Empedrado, 27°56′S, 58°45′W, Corrientes, Argentina 23 (M777492/CB198, topotype).

Ctenomys saltarius.—Tolombón, 26°11′S, 65°56′W, Salta, Argentina 15 (HM777493/CB295 or C-04689).

Ctenomys scagliai.—Tafí del Valle, 26°26′S, 65°57′W, Tucumán, Argentina 20 (HM777494/CB299 or C-04696, topotype).

Ctenomys sericeus.—La Porteña, Rio Lista, 48°02′S, 71°56′W, Santa Cruz, Argentina 61 (HM777496/sv45, topotype).

Ctenomys sociabilis.—Nahuel Huapi, 41°06′S, 71°18′W, Neuquén, Argentina 48 (HM777495/EAL 545, topotype).

Ctenomys steinbachi.—Buen Retiro, Bolivia 5 (AF007044).

Ctenomys talarum.—*talarum*1, El Guanaco, 36°32′S, 66°26′W, Buenos Aires, Argentina 43 (HM777497/315 or C-04773); *talarum*2 and *talarum*4, Necochea 38°33′S, 58°44′W, Buenos Aires, Argentina 46 (AF370698-9, topotype); *talarum*3 Saladillo, 35°38′S, 59°46′W, Buenos Aires, Argentina 45 (HM777498/IF1).

Ctenomys torquatus.—*torquatus*1, Ipora, Uruguay 33 (AF119111); *torquatus*2, Alegrete, Brazil 26 (EF372282).

Ctenomys tuconax.—El Infiernillo, Argentina 18 (AF370684).

Ctenomys tucumanus.—*tucumanus*1, Ticucho, Argentina 16 (AF370691); *tucumanus*2 San Miguel de Tucumán, 26°23′S, 64°41′W, Tucumán, Argentina 19 (HM777499/CB277 or C-04670, topotype).

Ctenomys sp.—sp1, Cerro Ventana, 42°01′S, 69°56′W, Chubut, Argentina 62 (HM777500/PNG803); sp2, La Paloma, 47°39′S, 67°46′W, Santa Cruz, Argentina, 59 (HM777501/LTU207); sp3, Cerro del Paso, 47°54′S, 66°23′W, Santa Cruz, Argentina 60 (HM777502/PNG1614); sp4, Pichiñan, 43°33′S, 69°04′W, Chubut, Argentina 55 (HM777503/PNG1201); sp5, Quichaura, 43°33′S 70°28′W, Chubut, Argentina 54 (HM777504/PNG336); sp6, Talagapa, 42°13′S, 68°16′W, Chubut, Argentina 52 (HM777505/PNG191); sp7, Somuncura, 41°26′S, 67°18′W, Río Negro, Argentina 53 (HM777506/PNG160).

Ctenomys sp. ITA.—ITA, Cerro Itahuaticua Tarija, Bolivia, 11 (AF007047).

Ctenomys sp. LLATHU.—LLATHU, Cochabamba, Bolivia, 3 (AF007048).

Ctenomys sp. MONTE.—MONTE, Monteagudo, Bolivia 8 (AF007053).

Ctenomys sp. MINUT.—MINUT, W of Robore, Bolivia 6 (AF007052).

Ctenomys sp.—ROBO, Robore, Bolivia 6 (AF007039).

Capromys.—AF422915, *Cavia*.—AY382791, *Coendou*.—AF411584, *Dactylomys*.—L23335, *Echimys*.—L23341, *Myoprocta*.—AF437781, *Octodon*.—AF007058, *Octodontomys*.—AF370706, *Proechimys*.—AJ251403, *Trinomys*.—AF422923, *Tympanoctomys*.—AF007060, *Bathyergus*, AY425911, *Thryonomys*.—NC002658.