Biological Journal of the Linnean Society (2000), *70:* 361–376. With 3 figures
doi:10.1006/bijl.1999.0394, available online at http://idealibrary.com on IDEAL®

Phylogenetics of the speciose and chromosomally variable rodent genus *Ctenomys* (Ctenomyidae, Octodontoidea), based on mitochondrial cytochrome *b* sequences

SILVIA MASCHERETTI[1] AND PATRICIA M. MIROL[2]

Department of Biology, University of York, PO Box No 373, York YO10 5YW

MABEL D. GIMÉNEZ AND CLAUDIO J. BIDAU

Departamento de Genética, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, 3300 Posadas, Misiones, Argentina

JULIO R. CONTRERAS

Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Av. Angel Gallardo 470, (1405) Buenos Aires, Argentina

JEREMY B. SEARLE[3]

Department of Biology, University of York, PO Box No 373, York YO10 5YW

Received 20 January 1999; accepted for publication 7 July 1999

The tuco-tucos (*Ctenomys*) are subterranean rodents that are widespread in southern South America. On the basis of its 60 living species, *Ctenomys* is one of the most speciose mammalian genera and displays great chromosomal variation. In order to study the mode of speciation in *Ctenomys* and to evaluate the role of chromosomes in cladogenesis, it is essential to generate an accurate phylogeny of the genus. From such a phylogeny it should be possible to identify particular species lineages worthy of further study. Following the success in 1998 of Lessa & Cook in generating a phylogeny of 10, mostly Bolivian, species from mitochondrial cytochrome *b* sequences, we sequenced a further 18, mostly Argentinian, species. By combining our

[1] Present address: Mucosal Immunology Group, 1st Department of Medicine, Christian-Albrechts University, Schittenhelmstr. 12, 24195 Kiel, Germany
[2] Present address: CIGEBA, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118 s/n, 1900 La Plata, Argentina
[3] Corresponding author. E-mail: jbs3@york.ac.uk

0024–4066/00/060361+16 $35.00/0

dataset with that of Lessa & Cook's we were able to confirm six species lineages within *Ctenomys*. These lineages correspond well to taxonomic groups suggested on the basis of morphology and biogeography. At least two of the species groups (the Boliviano-Matogrossense and Chaco) are very variable chromosomally, and are worthwhile systems to examine the possibility of chromosomal speciation. While, in general, multiple specimens of the named species did form monophyletic groups on the basis of the cytochrome *b* analysis, there were some exceptions. Thus, *C. bergi* and *C. yolandae* did not form separate clades in the phylogenetic analysis, nor did *C. roigi* and *C. dorbignyi*. The molecular analysis was also very informative with regards species designations for *C. boliviensis* and related forms.

ADDITIONAL KEYWORDS:—speciation – subterranean – tuco-tuco – chromosomal variation – Argentina – South America.

CONTENTS

Introduction 362
Material and methods 363
Specimens 363
DNA extraction and sequencing 364
Phylogenetic analysis 365
Results 366
Variability of the cytochrome *b* sequences 366
Phylogenetic analysis 367
Subsidiary analysis 367
Discussion 368
Pattern of molecular variation 368
Species limits in *Ctenomys* 370
Species groupings within *Ctenomys* 372
Acknowledgements 374
References 374

INTRODUCTION

Subterranean rodents show some of the most spectacular chromosomal variation in mammals. For example, *Thomomys* in North America, *Spalax* in the Middle East and *Ellobius* in Asia are all characterized by numerous different karyotypes (Thaeler, 1985; Savic & Nevo, 1990; Lyapunova *et al.*, 1980). The subterranean rodent genus *Ctenomys*, which is found in South America, conforms to the same pattern, with diploid numbers varying between 10 and 70 (Anderson *et al.*, 1987; Reig *et al.*, 1990). In *Ctenomys* much of this variation reflects differences between species; many of the 60 described species are karyotypically distinct (Reig *et al.*, 1990). The genus therefore represents an excellent model to examine the concept of chromosomal speciation: the idea that chromosomal rearrangements may promote speciation (King, 1993). However, preliminary to any detailed investigation of chromosomal speciation by survey work, breeding studies etc., there is an urgent need for the application of molecular methods to confirm and clarify the existing taxonomy. There have been a few useful allozyme studies (e.g. Sage *et al.*, 1986; Cook & Yates, 1994; Ortells & Barrantes, 1994), but DNA sequence analysis offers the opportunity to generate a clear, comprehensive phylogeny.

Lessa & Cook (1998) generated the first DNA phylogeny for *Ctenomys* on the basis of the complete cytochrome *b* sequences of 10 species, including all eight Bolivian

species recognized by Cook & Yates (1994). In this paper we extend this analysis to include a further 18 species, mostly from northern Argentina. Our analysis is based on a shorter length of cytochrome *b* sequence than that of the previous workers, but it has nevertheless yielded a robust phylogeny. Thus, in this paper, we are able to examine more critically than ever before the subdivision of the genus *Ctenomys* into species groups. Furthermore, we have also begun to use molecular data to examine species limits within the genus.

MATERIAL AND METHODS

Specimens

Specimens were collected during field trips over the period 1993–1996 and identified on the basis of external and cranial morphology and karyotype. The following 37 specimens were used for the main analysis (382 bp of cytochrome *b*). They were collected in Argentina (unless otherwise indicated), sometimes from the type locality (shown in bold), and have been deposited in the collection of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, Argentina, with the catalogue numbers shown: *C. argentinus* 1, 2 (C-04473, C-04474) Campo Winter, Chaco: 26°36′S 59°15′W (4 km from their type locality); *C. azarae* (C-04016) **General Acha, La Pampa**: 37°45′S 65°00′W; *C. bergi* 1 (C-03507) Salinas Grandes, Córdoba: 30°03′S 65°05′W; *C. boliviensis* 1 (C-03975) Santa Cruz de la Sierra, Santa Cruz, Bolivia: 17°48′S 63°10′W; *C. bonettoi* 1, 2, 3 (C-04465, C-04466, C-04467) **Colonia Elisa, Chaco**: 26°48′S 59°33′W; *C. dorbignyi* 1 (C-03691) Paso Vera, Entre Ríos: 32°29′S 58°14′W; *C. juris* (C-04709) **El Chaguaral, Jujuy**: 24°16′S 64°48′W; *C. latro* 1 (C-04678) Río Choromoro, Tucumán: 26°18′S 65°14′W; *C. latro* 2 (C-04679) **Tapia, Tucumán**: 26°36′S 65°18′W; *C. nattereri* 1, 2 (C-03968, C-03969) San Matías, Santa Cruz, Bolivia: 16°22′S 58°24′W; *C. nattereri* 3, 4, 5 (C-03970, C-03971, C-03972) Cascabel, Bolivia: 17°21′S 58°20′W; *C. occultus* 1, 2 (C-04685, C-04686) **Monteagudo, Tucumán**: 27°31′S 65°17′W; *C. opimus* 1 (C-04706) Tres Cruces, Jujuy: 22°54′S 65°35′W; *C. pilarensis* 1, 2 (C-03573, C03571) Mayor Martínez, Ñeembucú, Paraguay: 27°08′S 58°24′W; *C. pilarensis* 3 (C-03854) **Yatayty, Ñeembucú, Paraguay**: 26°52′S 58°13′W; *C. pilarensis* 4 (C-03572) Desmochado, Ñeembucú, Paraguay: 27°06′S 58°04′W; *C. pundti* 1 (C-04043) Puente Olmos, Córdoba: 32°28′S 63°20′W; *C. pundti* 2 (C-03753) La Carlota, Córdoba: 32°30′S 63°12′W; *C. roigi* 1 (C-03533) **Costa Mansión, Corrientes**: 28°08′S 58°49′W; *C. roigi* 2 (C-03842) Arroyo Peguajó, Corrientes: 28°10′S 59°49′W; *C. roigi* 3 (C-03749) Empedrado, Corrientes: 27°57′S 58°48′W; *C. scagliai* 1, 2 (C-04696, C-04697) **Route 307, km 95, Tucumán**: 26°38′S 65°49′W; *C. talarum* (C-04057) **Estancia La Florida, La Pampa**: 36°22′S 65°02′W; *C. tuconax* 1, 2 (C-04687, C-04688) La Calera, Tucumán: 27°20′S 63°35′W; *C. tucumanus* 1, 2 (C-04670, C-04671) **San Miguel de Tucumán, Tucumán**: 26°49′S 65°13′W; *C. yolandae* 1 (C-03581) Loteo Santa Ana, Santa Fe: 31°38′S 60°43′W.

Also included in the main analysis were the following *Ctenomys* from the GenBank database (Lessa & Cook, 1998): *C. boliviensis* 2, 3 (Accession number: AF007037, AF007038), *C. boliviensis* robo 1, 2 (AF007039, AF007040), *C. conoveri* 1, 2 (AF007054, AF007055), *C. frater* 1, 2 (AF007045, AF007046), *C. goodfellowi* 1, 2 (AF007050,

AF007051), *C. haigi* (AF007063), *C. leucodon* (AF007056), *C. lewisi* (AF007049), *C. mendocinus* (AF007062), *C. opimus* 2, 3 (AF007041, AF007042), *C. steinbachi* 1, 2 (AF007043, AF007044). The sequence data for *C. boliviensis* robo was only used in phylogeny construction; it was not used for other analyses.

The genus *Ctenomys* is the sole genus within the family Ctenomyidae, but within the superfamily Octodontoidea, the family Octodontidae represents a sister taxon (Nedbal *et al.*, 1994). Therefore, the three octodontids analysed by Lessa & Cook (1998) represent the most suitable outgroup taxa and were used as such in the main analysis: *Octodon degus* (Accession number: AF007058), *Spalacopus cyanus* (AF007061) and *Tympanoctomys barrerae* (AF007060).

A subsidiary analysis was conducted based on 260 bp of cytochrome *b*. This analysis made use of all the above specimens, plus the following 10 specimens: *C. bergi* 2 (C-03508) Guanaco Muerto, Córdoba: 30°28′S 65°03′W; *C. bergi* 3 (C-03506) Las Toscas, Córdoba: 30°09′S 65°54′W; *C. dorbignyi* 2, 3 (C-03817, C-03818) **Mbarigüi, Corrientes**: 27°33′S 57°31′W; *C. dorbignyi* 4, 5 (C-03685, C-03686) Paso Vera, Entre Ríos: 32°29′S 58°14′W; *C. roigi* 4 (C-03843) Arroyo Peguajó, Corrientes: 28°10′S 58°49′W; *C. rosendopascuali* 1, 2 (C-03509, C-03510) Los Mistoles, Córdoba: 30°38′S 63°43′W; *C. yolandae* 2 (C-03581) Loteo Santa Ana, Santa Fe: 31°38′S 60°43′W.

Figure 1 shows the collection sites for all specimens analysed.

DNA extraction and sequencing

DNA was extracted from tail tissue conserved in 100% ethanol by using three units of proteinase K in 0.5 ml of Tris-EDTA-SDS extraction buffer. A mix of phenol:chloroform:isoamyl alcohol (25:24:1) was used to extract proteins and the DNA was precipitated with an equal volume of isopropanol and 1/10 volume of 5M ammonium acetate.

For the 37 new specimens for the main analysis, a 425 bp fragment of the mitochondrial cytochrome *b* gene was amplified using the following primers: L14724 and H15149 (Irwin *et al.*, 1991). The PCR amplification cycle consisted of 1 min at 93°C, 1 min at 50°C and 2 min at 72°C, repeated 30 times. PCR products were purified using the Wizard PCR Preps kit (Promega). A 382 bp sequence of cytochrome *b* was obtained with the L14724 primer, using a Taq DyeDeoxy Terminator cycle Sequencing kit (ABI Applied Biosystems) before loading onto an ABI 373 automated sequencer.

We could be sure that this was the correct mt DNA sequence for the following reasons: (a) for all individuals we obtained a single PCR band which produced a clean direct sequence, (b) the same sequence was produced using the other primer for those several individuals tested, (c) test studies also showed that different overlapping primers produced the same sequence, (d) extension of the sequence in two individuals (*C. latro* 1 and *C. tucumanus* 1) generated a complete, typical, functional cytochrome *b* gene, (e) for all individuals the sequence had similarity to those sequences of *Ctenomys* already produced independently by Lessa & Cook (1998).

The 10 specimens used for the subsidiary analysis arose from a study of nuclear pseudogenes generated by the primers L14841 and H15149 (Kocher *et al.*, 1989). The methodology and pseudogene sequences generated will be reported in detail elsewhere (Mirol *et al.*, 2000). The sequences that we report here were produced

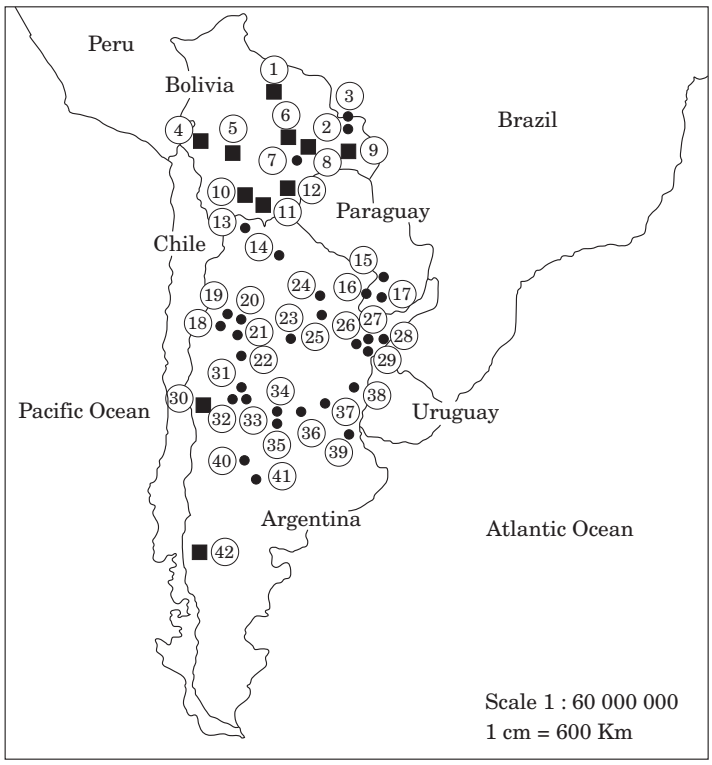

Figure 1. Collection localities of all 47 *Ctenomys* that were sequenced by us (●) and the 18 *Ctenomys* from the GenBank databases sequenced by Lessa & Cook (1998) (■). Key to specimens (see Materials and Methods): Localities in Bolivia: (1) *C. goodfellowi* 1, 2; (2) *C. nattereri* 1, 2; (3) *C. nattereri* 3, 4, 5; (4) *C. leucodon*; (5) *C. opimus* 2, 3; (6) *C. steinbachi* 1, 2; (7) *C. boliviensis* 1; (8) *C. boliviensis* 2, 3; (9) *C. boliviensis* robo 1, 2; (10) *C. lewisi*; (11) *C. frater* 1, 2; (12) *C. conoveri* 1, 2; Localities in Argentina and Paraguay: (13) *C. opimus* 1; (14) *C. juris*; (15) *C. pilarensis* 1, 2; (16) *C. pilarensis* 3; (17) *C. pilarensis* 4; (18) *C. tucumanus* 1, 2; (19) *C. latro* 1; (20) *C. latro* 2; (21) *C. scagliai* 1, 2; (22) *C. occultus* 1, 2; (23) *C. tuconax* 1, 2; (24) *C. bonettoi* 1, 2, 3; (25) *C. argentinus* 1, 2; (26) *C. roigi* 1; (27) *C. roigi* 2; (28) *C. roigi* 3; (29) *C. roigi* 3; (30) *C. mendocinus*; (31) *C. bergi* 1; (32) *C. bergi* 2; (33) *C. bergi* 3; (34) *C. pundti* 1; (35) *C. pundti* 2; (36) *C. rosendopascuali* 1, 2; (37) *C. yolandae* 1, 2; (38) *C. dorbignyi* 1, 4, 5; (39) *C. dorbignyi* 2, 3; (40) *C. talarum*; (41) *C. azarae*; (42) *C. haigi*.

after cloning and are believed to be true mtDNA sequences because of their close similarity or identity to sequences produced in the main analysis and because they showed appropriate levels of between individual variation.

All our sequences have been deposited in the GenBank database (accession numbers: AF 143212–143221, AF 144263–144299).

Phylogenetic analysis

The sequences were aligned using SeqEd (ABI) and PILEUP (Program Manual for the Wisconsin Package, version 8, 1994). The transition/transversion characteristics of the sequences and the translations into amino acid sequence were obtained using MacClade (Maddison & Maddison, 1992).

To evaluate the phylogenetic information of the dataset the distribution of 10 000 randomly generated trees was assessed for skewness (the g1 test), as detailed by Hillis (1991) and Hillis & Huelsenbeck (1992), using PAUP Version 3.1 (Swofford, 1993).

Maximum Parsimony analyses were run using PAUP under different weighting schemes: the three codon positions were unweighted or there was a 2:5:1 ratio for the first, second and third positions; transversions were unweighted or allocated 5, 10 and 20 times more than transitions. The shortest tree(s) were found in a heuristic search with 10 replicates of 100 random stepwise addition of taxa. Where more than one minimum length tree was found, a strict consensus tree was produced. To generate bootstrap values for the parsimony trees, separate analyses were conducted based on 100 pseudoreplicates, using 10 random orders of input taxa.

Phylogenetic analysis was also conducted using the PHYLIP package (Felsenstein, 1991). Pairwise distances between taxa were calculated using DNADIST, under the assumptions of the Kimura 2-parameter model. Distance trees were constructed using the Neighbour-joining method (Saitou & Nei, 1987) and the SEQBOOT program was used to obtain bootstrap values from 1000 pseudoreplicates of the data set. The data were either unweighted or transversions were weighted 5, 10 and 20 times more than transitions.

RESULTS

Variability of the cytochrome b *sequences*

Considering the main dataset, comprising our own data and published data of Lessa & Cook (1998), a total of 53 specimens of *Ctenomys* belonging to 27 species were available for comparison. Based on the 382 bp sequence available for all specimens, a total of 125 variable sites was recorded (33% of nucleotides). The large majority of substitutions recorded were transitions (103 out of 125 [82%]) and there was a clear bias towards third position changes (24 [19%] 1st: 6 [4%] 2nd: 95 [76%] 3rd). All second position and most (21[88%]) first position substitutions led to a change in amino acids, while all third position substitutions were silent. Thus, there were a total of 27 amino acid changes (at 26 positions) out of the 127 amino acids considered. As previously observed for other mammals (Irwin *et al.*, 1991), including the closely related echimyids (Lara *et al.*, 1996), the amino acid changes were mostly localized in the transmembrane domains.

In several cases it was possible to examine the intraspecific variation in DNA sequence. For the following species studied by us and Lessa & Cook (1998) there are data for two individuals from one population: *C. argentinus, C. boliviensis, C. conoveri, C. frater, C. goodfellowi, C. occultus, C. opimus, C. pilarensis, C. scagliai, C. steinbachi, C. tuconax, C. tucumanus*. For *C. bonettoi* there are data for three individuals from one population and for *C. nattereri* there are data for two individuals from one population and three individuals from another. In the majority of comparisons, the haplotypes were the same for individuals from a single population. The two individuals of *C. steinbachi, C. tuconax* and *C. goodfellowi* differed from each other by 1, 4 and 6 nucleotides, respectively. Thus, for 19 within population comparisons (15 populations, 14 species) there was a mean pairwise distance of 0.58 nucleotides (0.2% of the cytochrome *b* fragment examined). In contrast, 17 between population comparisons

for *C. boliviensis*, *C. latro*, *C. nattereri*, *C. opimus*, *C. pilarensis*, *C. pundti* and *C. roigi* yielded a mean pairwise distance of 1.94 nucleotides (0.5% of the cytochrome *b* fragment examined), with a range of 0–7 nucleotides.

The molecular divergence between species varied enormously. *C. yolandae* 1 and *C. bergi* 1 shared the same haplotype, while the maximum divergence of 48 nucleotides (i.e. 12.6% divergence of the sequence) was between *C. leucodon* and *C. tuconax* 1.

Phylogenetic analysis

The g1 test for phylogenetic information yielded highly significant results for all the weighting schemes applied and, indeed, whatever the weighting, the parsimony and distance methods yielded similar trees with reasonable degree of structure (Fig. 2).

The following lineages can be identified from these different analyses:

(a) Lessa and Cook (1998) found that *C. conoveri*, *C. frater* and *C. lewisi* formed a lineage with high bootstrap support. These species also group together in all our trees, but with less confidence, perhaps reflecting the shorter sequence under consideration.

(b) The strong relationship of *C. boliviensis* and *C. goodfellowi* demonstrated by Lessa and Cook (1998) is still supported in our trees after addition of a new haplotype. In the distance tree, we found a linkage (60% bootstrap support) of the *boliviensis–goodfellowi* lineage with another lineage that we consistently found to be well supported: that between *C. nattereri* and *C. boliviensis* robo. There are also indications from this tree that *C. steinbachi* forms part of this species grouping.

(c) The grouping of *C. argentinus*, *C. latro*, *C. occultus* and *C. pilarensis* is well-supported in all our analyses, and it appears that *C. juris* is also related to these four species.

(d) *C. bergi*, *C. bonettoi* and *C. yolandae* form a well-supported group which are apparently also related to *C. azarae* and *C. mendocinus*.

(e) *C. pundti* and *C. talarum* form a strongly supported lineage.

(f) *C. dorbignyi* and *C. roigi* form a strongly supported lineage.

There are no well-supported relationships among these six lineages. Also, *C. haigi*, *C. leucodon*, *C. opimus*, *C. scagliai*, *C. tuconax* and *C. tucumanus* are not clearly affiliated to any other species.

Subsidiary analysis

The subsidiary analysis was based on a 260 bp cytochrome *b* sequence from a total of 65 specimens belonging to 28 species of *Ctenomys*. Parsimony and distance trees were obtained, with little variation between them. Figure 3 shows part of a parsimony tree showing those data not included in the main analysis. The new species, *C. rosendopascuali*, appears to be another member of the *azarae–bergi–bonettoi–mendocinus–yolandae* lineage already identified. The new data on *C. bergi* and *C. yolandae* reinforce the previous observation of their similarity in terms of cytochrome *b* sequence: the named species do not form monophyletic groups on the basis of the molecular data.

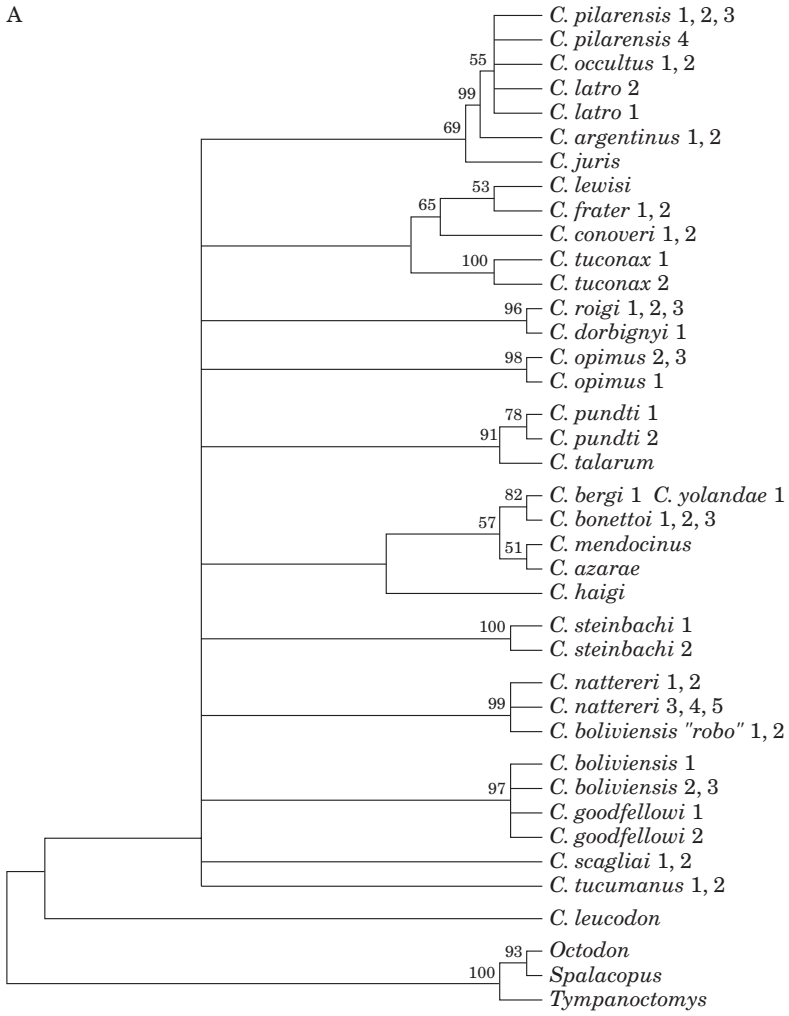

Figure 2. Caption on facing page.

The new data on *C. dorbignyi* and *C. roigi* also reveal uncertainty of species status within their lineage.

DISCUSSION

Pattern of molecular variation

The most important issue to address is whether the sequence variation described is appropriate to generate a meaningful phylogeny of *Ctenomys*. As already stated, the characteristics of the sequence analysed are as expected of a functional mitochondrial gene. The length of sequence analysed is only 382 base pairs for the main analysis and 260 for the subsidiary analysis. However, a very large number of

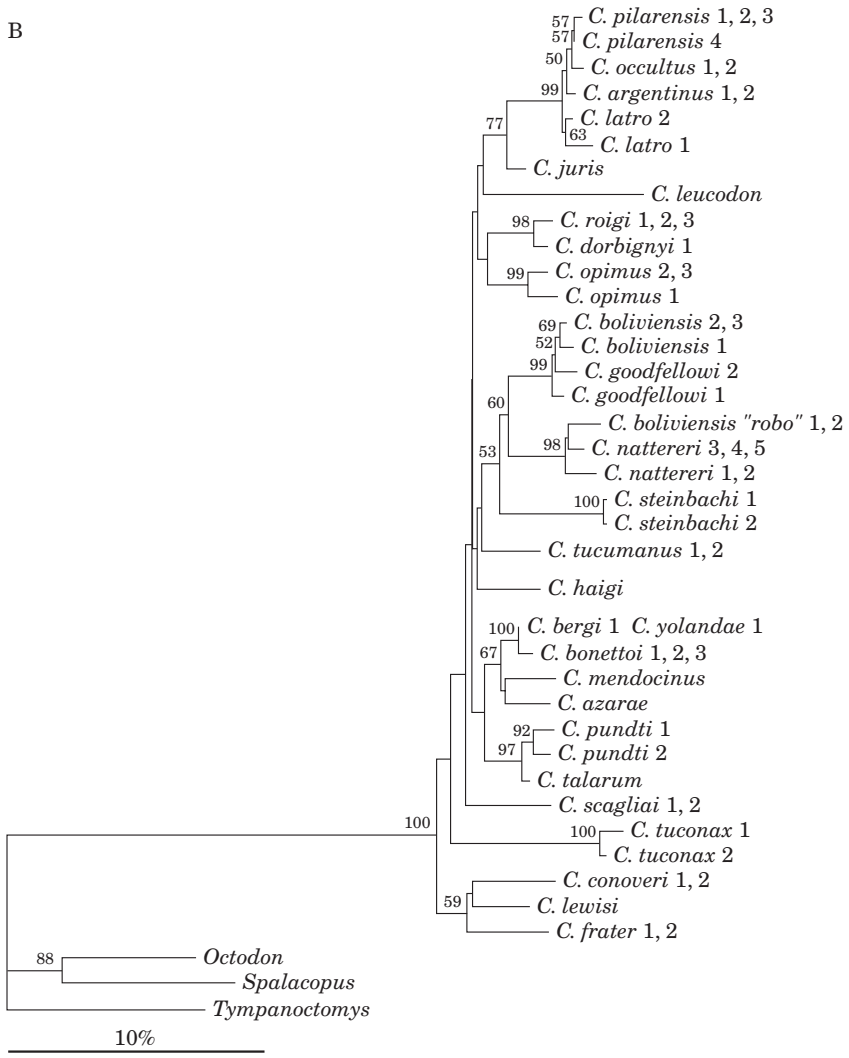

Figure 2. Unweighted phylogenetic trees of species of *Ctenomys*, based on 382 base pairs of cytochrome *b* sequence (including sequences taken from Lessa & Cook, 1998) with *Octodon*, *Spalacopus* and *Tympanoctomys* as outgroups (Lessa & Cook, 1998). A (facing page), strict consensus of four equally parsimonious trees (412 steps long and CI = 0.493) obtained with PAUP. Bootstrap values are given as percentages and represent 100 pseudoreplicates. B, Neighbour-joining tree from a Kimura 2-parameter distance matrix using PHYLIP. Bootstrap values are given as percentages and represent 1000 pseudoreplicates.

nucleotide changes were detected in this sequence. Because of this copious variation and the large-scale sharing substitutions between species, the phylogenies that we generated included lineages with strong bootstrap support.

These phylogenies are based very substantially on third position transitions which may be problematical if such transitions have reached saturation for the taxa under consideration. Such a situation could create a large degree of homoplasy capable of obscuring the phylogeny. In order to test this, the number of third position

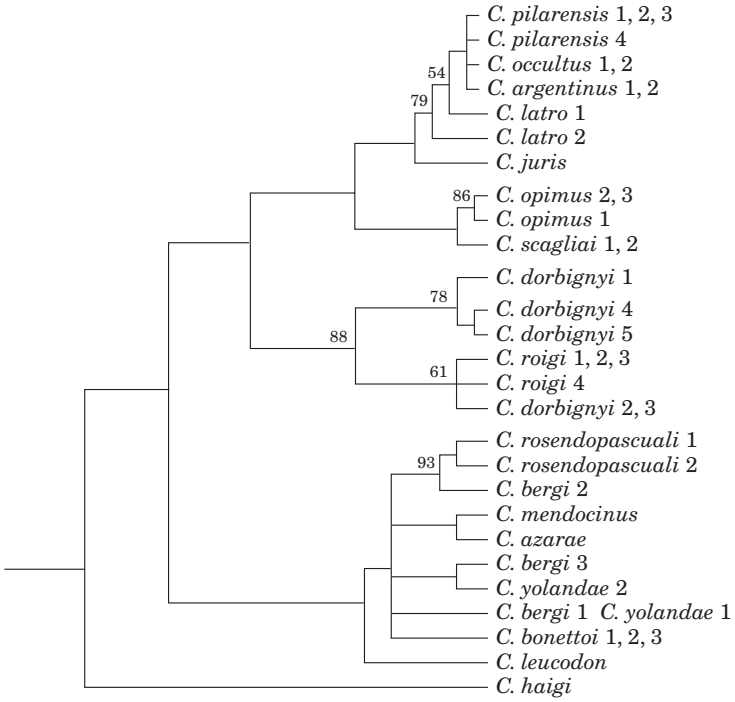

Figure 3. Part of a unweighted strict consensus parsimony tree based on 20 equally parsimonious trees (281 steps long and CI = 0.534) obtained with PAUP and based on an analysis of 65 individuals of 28 species of *Ctenomys* (with the same outgroup species as in Fig. 2) for a 260 base pair fragment of cytochrome *b*. This part of the tree includes all those specimens not included in Fig. 2. Bootstrap values are given as percentages and represent 100 pseudoreplicates.

transitions was plotted against the maximum likelihood distance among the taxa. (The maximum likelihood distances were obtained using the DNAML program within the PHYLIP package with empirical base composition biases and unweighted data.) An asymptote was not reached, indicating saturation is not a serious issue. This accords well with a similar analysis by Lara *et al.* (1996) on the Echimyidae, which are South American rodents closely allied to *Ctenomys*. They found saturation occurred when there was a sequence divergence of about 20%. Among the *Ctenomys*, the most distant species in terms of cytochrome *b* sequence differed by 13%.

Although our dataset is adequate to generate a phylogeny of *Ctenomys*, we have not attempted to date branch points, given the uncertainties of molecular clocks and the shortness of our sequence.

Species limits in Ctenomys

For most species of *Ctenomys* for which several individuals were sequenced, the different individuals had the same or closely similar haplotypes. There was more nucleotide divergence between individuals collected in different geographic locations than between individuals within populations. Clearly, future studies should examine this in more detail. Given that new species are ultimately the product of within species

variation, an understanding of this will be important to explain the phenomenal diversification of the genus.

There are a number of species for which the different specimens examined failed to form a monophyletic group within the trees that we generated. The most dramatic situation involves the *boliviensis–goodfellowi–nattereri–boliviensis* robo lineage (Fig. 2B). Lessa & Cook (1998) generated the *C. boliviensis* robo sequence and have already suggested that it may represent a different species from the typical *C. boliviensis*. This appears very likely, as there is a substantial nucleotide divergence between the *C. boliviensis* and *C. boliviensis* robo haplotypes (19–20 out of the 382 nucleotides analysed; approximately 5%). Instead there is a much smaller divergence between the *C. boliviensis* robo and *C. nattereri* haplotypes (7 and 10 nucleotides; approximately 2%), similar to that between the different haplotypes of *C. nattereri* (7 nucleotides). Also, as was clear already in the phylogenetic trees of Lessa & Cook (1998), *C. goodfellowi* is paraphyletic and cannot be distinguished from *C. boliviensis* on the basis of the cytochrome *b* sequence.

Anderson *et al.* (1987) considered all these taxa as forms of *C. boliviensis* from morphology, with *C. nattereri* classified as *C. boliviensis nattereri* and *C. goodfellowi* as *C. boliviensis goodfellowi*. However, Cook & Yates (1994) argued that *goodfellowi* should be given specific status and *nattereri* was originally described as a separate species by Wagner (1848). *C. boliviensis* robo was classified as typical *C. boliviensis* (i.e. *C. boliviensis boliviensis*) by Anderson *et al.* (1987).

The molecular data suggest that *goodfellowi* should be considered conspecific with typical *C. boliviensis*. In fact the chromosome number is similar ($2n = 46$ and 44, respectively: Anderson *et al.*, 1987) and allozyme differentiation is small (Cook & Yates, 1994).

In contrast, the molecular data suggest that *C. boliviensis* robo may more properly be incorporated within *C. nattereri*, although more morphological, chromosomal and molecular studies are essential to clarify this particular linkage.

It would also be worth conducting further studies on the species status of *C. bergi* and *C. yolandae*. The specimens sequenced over 382 base pairs had identical haplotypes. Further related haplotypes were found in the subsidiary analysis (Fig. 3). The diploid chromosome numbers of the two forms are similar with 48 for *C. bergi* and 50 for *C. yolandae* (Ortells *et al.*, 1990: Reig *et al.*, 1990).

In the subsidiary analysis (Fig. 3) there were another two forms previously described as species, which did not form separate clades: *C. roigi* and *C. dorbignyi*. *C. roigi* is a highly endangered and localized form (Contreras & Bidau, 1999). Its close relation with *C. dorbignyi* is particularly interesting given a large difference in chromosomal number (*C. dorbignyi*: $2n = 70$, *C. roigi*: $2n = 48$) (Ortells *et al.*, 1990). Although the ancestral chromosome number in *Ctenomys* is not confirmed, diploid numbers of 48 and 50 are particularly prevalent (Giménez *et al.*, 1999). Therefore, *C. roigi* may represent a relict of the ancestral condition, with *C. dorbignyi* a more widespread derived form, produced by numerous chromosomal fissions.

Although the molecular phylogenies are providing much interesting information, we need to be cautious about redefining species limits on the basis of such data (Harrison, 1991). On the biological species concept, reproductive isolation should be the defining characteristic to distinguish species. The difficulties with trying to relate the molecular data to species limits are exemplified by studies of *Thomomys*, the subterranean rodent genus in North America (Patton & Smith, 1994; Smith, 1998). In this genus, unlike *Ctenomys*, there have been extensive studies of natural

hybridization, as well as phylogenetic analysis of cytochrome *b* and other molecular markers. *T. bottae* and *T. townsendii* are defined as separate biological species because hybridization is essentially limited to the F_1 generation in hybrid zones while there is no evidence of reproductive isolation between geographic units within either species. However, *T. townsendii* cytochrome *b* haplotypes not only fail to form a monophyletic lineage, they are found within one of several distinct *T. bottae* lineages each of which relates to a different geographic unit of *T. bottae*. Therefore, the lack of clear separation on the basis of cytochrome *b* between, for instance, *C. bergi* and *C. yolandae* does not necessarily rule out the possibility that they are good biological species. Furthermore, there is also the possibility that some of the so-called species which form separate cytochrome *b* clades in our study, may, in fact, be conspecific with taxa elsewhere in the tree.

Species groupings within Ctenomys

The main aim of this study was to identify species groupings within the genus *Ctenomys* to compare with species groups already proposed on the basis of biogeography and morphology. In order to understand the phenomenal diversification of the genus, it is necessary to subdivide it into more manageable groups of species in which chromosomal evolution, range changes and other features can be documented.

For the Argentinian and Bolivian species that we have examined, there is already a suggested series of species groups based on biogeography and morphological similarity (Contreras & Bidau, 1999). Several of these groups were well-represented in the molecular studies and it was found that the molecular data very substantially support these lineages (Table 1). Particularly notable groups are the Boliviano-Matogrossense group, the Chaco group and the *mendocinus* complex/Eastern group for which there are cytochrome *b* sequence data for 5, 6 and 7 taxa, respectively. Given the desire to use the molecular data to identify those representatives of the genus *Ctenomys* that are most interesting from the viewpoint of chromosomal variation, we discuss this aspect for each group in turn.

Boliviano-Matogrossense group. This group forms a monophyletic lineage in the molecular analysis. We have already discussed the relationship of *C. goodfellowi*, *C. boliviensis*, *C. boliviensis* robo and *C. nattereri*. *C. boliviensis* including *goodfellowi*, is apparently subdivided into several chromosomal races ($2n = 42$, 44, 46: Anderson *et al.*, 1987) while *C. boliviensis* robo has a distinctly different karyotype ($2n = 36$: Anderson *et al.*, 1987), which we would predict is similar to *C. nattereri*, which has not been karyotyped. Thus, this is a particularly interesting group to study, with both incipient chromosomal species (i.e. the chromosomal races of *C. boliviensis*) and the putative products of chromosomal speciation, most notably *C. steinbachi* which has a diploid number of 10 (Anderson *et al.*, 1987), much below other members of the group. *C. steinbachi* could have arisen by multiple chromosomal fusions in an analogous fashion to the muntjac (Neitzel, 1987) and certain chromosomal races of the house mouse (Sage *et al.*, 1993)

Chaco group. Although this group is not entirely supported by the molecular analysis, *C. argentinus*, *C. latro*, *C. occultus* and *C. pilarensis* are clearly closely related. As for the Boliviano-Matogrossense group, there is both within species chromosomal variation

TABLE 1. Comparison of phylogenetic groups of *Ctenomys* proposed on the basis of morphology/biogeography (Contreras & Bidau, 1999) and cytochrome *b* sequence

Named morphological/ biogeographical group	Distribution	Representatives of group studied by molecular analysis	Interpretation of molecular data
Boliviano-Matogrossense	Bolivia	*boliviensis* *boliviensis* robo *goodfellowi* *nattereri* *steinbachi*	proposed lineage
Boliviano-Paraguayo	Bolivia, Paraguay	*conoveri* *frater* *lewisi*	proposed lineage
Chaco	Chaco Tucumán Paraguay	*argentinus* *latro* *occultus* *pilarensis* *scagliai* *tucumanus*	*arg.-lat.-occ.-pil.* form a proposed lineage; other species have uncertain affinity
mendocinus complex	La Pampa Córdoba Rio Negro Mendoza	*azarae* *bergi* *haigi* *mendocinus*	*aza.-ber.-men.* are part of a proposed lineage with the eastern group; *haigi* is distinct
Eastern	Chaco Córdoba Santa Fe	*bonettoi* *rosendopascuali* *yolandae*	all join with *aza.-ber.-men.* to form a proposed lineage
Ancestral group	Córdoba	*pundti*	proposed lineage
(unnamed)	La Pampa	*talarum*	
Corrientes	Corrientes Entre Rios	*dorbignyi* *roigi*	proposed lineage
Other species	Various	*juris* *leucodon* *opimus* *tuconax*	uncertain affinity, except *juris* which is associated with the *arg.-lat.-occ.-pil.* lineage

(e.g. *C. pilarensis*: Giménez *et al.*, 1997) and extreme between species variation. Thus, *C. occultus* has a diploid number of $2n = 22$, while the other species vary between $2n = 40$–50 (Reig & Kiblisky, 1969; Ortells *et al.*, 1990; Giménez *et al.*, 1997). Again, this appears to be an example of multiple chromosomal fusions.

Mendocinus *complex/Eastern group*. The *mendocinus* complex and Eastern group have been considered two separate, though related, lineages on the basis of features such as sperm symmetry. However, there are authors who have linked these two groups together (e.g. Freitas, 1994) and this is clearly supported by the molecular phylogeny. Chromosome number does not vary substantially among the species found in the *mendocinus* complex and Eastern group; $2n = 46$–52 for the six species identified within the molecular lineage (*C. azarae, C. bergi, C. mendocinus, C. bonettoi, C. rosendopascuali*

and *C. yolandae*) (Reig & Kiblisky, 1969; Ortells *et al.*, 1990; Giménez *et al.*, 1999; Massarini *et al.*, 1998). Although there is some interesting within species variation, in general this group does not appear to be promising with regards studies of chromosomal speciation.

Thus, the molecular analysis has produced an encouraging picture with regards the value of the genus *Ctenomys* in the study of chromosomal speciation. It has confirmed that there are at least two lineages within the genus (the Boliviano-Matogrossense group and the Chaco group) where both substantial within and between species variation in chromosome number occur. Also there is molecular support for the close relationship of *C. dorbignyi* and *C. roigi*, presumably as part of the Corrientes group (Table 1). As described above, these two species differ substantially in chromosome number. Clearly, future molecular studies are desirable to establish the full range of species within each of the phylogenetic groups within *Ctenomys*. Then a range of cytogenetic and reproductive studies need to be carried out to assess the role of chromosomes in the diversification of the genus. In this way *Ctenomys* may become a new model to complement systems such as the chromosomal races of *Mus musculus domesticus* (Sage *et al.*, 1993) or chromosomal races and chromosomally-distinct species of the *Sorex araneus* species complex (Searle & Wójcik, 1998; Zima *et al.*, 1998).

Although a number of species groups have been identified, nothing can be said about the relationships of those groups (with the exception of the *mendocinus* complex and Eastern group, which, as we have indicated should be considered a single group). Thus, our data are consistent with the contention of Lessa & Cook (1998) that there was an rapid star-like radiation of the genus *Ctenomys* soon after formation and that all the major lineages were formed at this time. However, the addition of more species may change the picture. It is clear that there is currently active cladogenesis within the genus and given more species and a greater length of sequence a phylogenetic signal may be uncovered for the period between early diversification of the genus and recent speciation events.

ACKNOWLEDGEMENTS

We are grateful to Drs David Bilton, Joe Cook and Maarit Jaarola for very useful comments and discussions. Yolanda Davies helped with collection of material. This study was supported by Fundación Antorchas, the British Council, CONICET, and grant BID 802/OC-ARPICT 01-00000-01205 (FONCYT) to CJB.

REFERENCES

Anderson S, Yates TL, Cook JA. 1987. Notes on Bolivian mammals 4. The genus *Ctenomys* (Rodentia, Ctenomyidae) in the eastern lowlands. *American Museum Novitates* **2891:** 1–20.

Contreras JR, Bidau CJ. 1999. Lineas Generales del Panorama Evolutivo de los Roedores Excavadores Sudamericanos del genero *Ctenomys* (Mammalia, Rodentia, Caviomorpha: Ctenomyidae). Ciencia Siglo XXI, 1: 1–22, Fundación Bartolomé Hidalgo, Buenos Aires.

Cook JA, Yates TL. 1994. Systematic relationships of the Bolivian tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). *Journal of Mammalogy* **75:** 583–599.

Felsenstein J. 1991. *PHYLIP (phylogeny inference package), version 3.4*. Department of Genetics, University of Washington, Seattle.

Freitas TRO de. 1994. Geographical variation of heterochromatin in *Ctenomys flamarioni* (Rodentia-Octodontidae) and its cytogenetic relationship with other species of the genus. *Cytogenetics and Cell Genetics* **67:** 193–198.

Giménez MD, Contreras JR, Bidau CJ. 1997. Chromosomal variation in *Ctenomys pilarensis*, a recently described species from eastern Paraguay (Rodentia, Ctenomyidae). *Mammalia* **61:** 385–398.

Giménez MD, Bidau CJ, Argüelles CF, Contreras JR. 1999. Chromosomal characterization and relationship between two new species of *Ctenomys* (Rodentia, Ctenomyidae) from northern Cordoba province, Argentina. *Zeitschrift für Säugetierkunde* **44:** 91–106.

Harrison RG. 1991. Molecular change at speciation. *Annual Review of Ecology and Systematics* **22:** 281–308.

Hillis DM. 1991. Discriminating between phylogenetic signal and random noise in DNA sequences. In: Miyamoto MM, Cracraft J, eds. *Phylogenetic analysis of DNA sequences*. New York: Oxford University Press, 278–294.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analysis. *Journal of Heredity* **83:** 189–195.

Irwin DM, Kocher TD, Wilson AC. 1991. Evolution of the cytochrome *b* of mammals. *Journal of Molecular Evolution* **32:** 128–144.

King M. 1993. *Species evolution: the role of chromosome change*. Cambridge: Cambridge University Press.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences USA* **86:** 6196–6200.

Lara MC, Patton JL, da Silva MNF. 1996. The simultaneous diversification of South American echimyid rodents (Hystricognathi) based on complete cytochrome *b* sequences. *Molecular Phylogenetics and Evolution* **5:** 403–413.

Lessa EP, Cook JA. 1998. The molecular phylogenetics of tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae) suggests an early burst of speciation. *Molecular Phylogenetics and Evolution* **9:** 88–99.

Lyapunova EA, Vorontsov NN, Korobitsyna KV, Ivanitskaya EY, Borisov YM, Yakimenko LV, Dovgal VY. 1980. A Robertsonian fan in *Ellobius talpinus*. *Genetica* **52/53:** 239–247.

Maddison WP, Maddison DR. 1992. *MacClade, version 3*. Sunderland, Massachusetts: Sinauer.

Massarini AI, Dyzenchauz FJ, Tiranti SI. 1998. Geographic variation of chromosomal polymorphism in nine populations of *Ctenomys azarae*, tuco-tucos of the *Ctenomys mendocinus* group (Rodentia: Octodontidae). *Hereditas* **128:** 207–211.

Mirol PM, Mascheretti S, Searle JB. 2000. Multiple nuclear pseudogenes of mitochondrial cytochrome *b* in *Ctenomys* (Caviomorpha, Rodentia) with either great similarity to or high divergence from the true mitochondrial sequence. *Heredity* **84**.

Nedbal MA, Allard MW, Honeycutt RL. 1994. Molecular systematics of hystricognath rodents: evidence from the mitochondrial 12S rRNA gene. *Molecular Phylogenetics and Evolution* **3:** 206–220.

Neitzel H. 1987. Chromosome evolution of Cervidae: karyotypic and molecular aspects. In: Obe G, Basler A, eds. *Cytogenetics*. Berlin: Springer-Verlag, 90–112.

Ortells MO, Contreras JR, Reig OA. 1990. New *Ctenomys* karyotypes (Rodentia, Octodontidae) from north-eastern Argentina and from Paraguay confirm the extreme chromosomal multiformity of the genus. *Genetica* **82:** 189–201.

Ortells MO, Barrantes G. 1994. A study of genetic distances and variability in several species of the genus *Ctenomys* (Rodentia: Octodontidae) with special reference to a probable causal role of chromosomes in speciation. *Biological Journal of the Linnean Society* **53:** 189–208.

Patton JL, Smith MF. 1994. Paraphyly, polyphyly, and the nature of species boundaries in pocket gophers (genus *Thomomys*). *Systematic Biology* **43:** 11–26.

Program Manual of the Wisconsin Package, Version 8. 1994. Genetics Computer Group, 575 Science Drive, Madison, Wisconsin.

Reig OA, Kiblisky P. 1969. Chromosome multiformity in the genus *Ctenomys* (Rodentia, Octodontidae). *Chromosoma* **28:** 211–244.

Reig OA, Busch C, Ortells MO, Contreras JR. 1990. An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. In: Nevo E, Reig OA, eds. *Evolution of subterranean mammals at the organismal and molecular levels*. New York: Alan R. Liss, 71–96.

Sage RD, Contreras JR, Roig VG, Patton JL. 1986. Genetic variation in South American

burrowing rodents of the genus *Ctenomys* (Rodentia: Ctenomyidae). *Zeitschrift für Säugetierkunde* **51:** 158–172.

Sage RD, Atchley WR, Capanna E. 1993. House mice as models in systematic biology. *Systematic Biology* **42:** 523–561.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

Savic IR, Nevo E. 1990. The Spalacidae: evolutionary history, speciation, and population biology. In: Nevo E, Reig OA, eds. *Evolution of subterranean mammals at the organismal and molecular levels*. New York: Alan R. Liss, 129–143.

Searle JB, Wójcik JM. 1998. Chromosomal evolution: the case of *Sorex araneus*. In: Wójcik JM, Wolsan M, eds. *Evolution of shrews*. Bialowieza, Poland: Mammal Research Institute, 219–268.

Smith MF. 1998. Phylogenetic relationships and geographic structure in pocket gophers in the genus *Thomomys*. *Molecular Phylogenetics and Evolution* **9:** 1–14.

Swofford DL. 1993. *Paup: phylogenetic analysis using parsimony, version 3.1*. Campaign, Illinois: Illinois Natural History Survey.

Thaeler CS jnr. 1985. Chromosome variation in the *Thomomys talpoides* complex. *Acta Zoologica Fennica* **170:** 15–18.

Wagner A. 1848. Beitrage zur Kenntnis der Arten von *Ctenomys*. *Arch. Naturg. Jahrg.* **14:** 72–78.

Zima J, Lukácová L, Macholán M. 1998. Chromosomal evolution in shrews. In: Wójcik JM, Wolsan M, eds. *Evolution of shrews*. Bialowieza, Poland: Mammal Research Institute, 175–218.