Evolution and phylogenetic relationships in subterranean rodents of Ctenomys mendocinus species complex: effects…

Article · August 2017

DOI: 10.1016/j.mambio.2017.08.002

Fernando Mapelli
Universidad Nacional de Mar del Plata
15 PUBLICATIONS **92** CITATIONS
SEE PROFILE

Matías Sebastián Mora
Instituto de Investigaciones Marinas y Costeras…
83 PUBLICATIONS **370** CITATIONS
SEE PROFILE

Maria Jimena Gomez Fernandez
Museo Argentino de Ciencias Naturales "Bernar…
9 PUBLICATIONS **15** CITATIONS
SEE PROFILE

Marcelo J Kittlein
Universidad Nacional de Mar del Plata
71 PUBLICATIONS **509** CITATIONS
SEE PROFILE

Title: Evolution and phylogenetic relationships in subterranean rodents of *Ctenomys mendocinus* species complex: effects of Late Quaternary landscape changes of Central Argentina

Authors: Fernando J. Mapelli, Matías S. Mora, Juan P. Lancia, María J. Gómez Férnandez, Patricia M. Mirol, Marcelo J. Kittlein

PII: S1616-5047(16)30219-1
DOI: http://dx.doi.org/doi:10.1016/j.mambio.2017.08.002
Reference: MAMBIO 40925

To appear in:

Received date: 27-12-2016
Accepted date: 4-8-2017

Please cite this article as: Mapelli, Fernando J., Mora, Matías S., Lancia, Juan P., Gómez Férnandez, María J., Mirol, Patricia M., Kittlein, Marcelo J., Evolution and phylogenetic relationships in subterranean rodents of Ctenomys mendocinus species complex: effects of Late Quaternary landscape changes of Central Argentina.Mammalian Biology http://dx.doi.org/10.1016/j.mambio.2017.08.002

Title: Evolution and phylogenetic relationships in subterranean rodents of *Ctenomys mendocinus* species complex: effects of Late Quaternary landscape changes of Central Argentina

Authors: Fernando J. Mapelli[1, 2,*], Matías S. Mora[1], Juan P. Lancia [1,] María J. Gómez Férnandez[2], Patricia M. Mirol[2], and Marcelo J. Kittlein[1]

[1]Instituto de Investigaciones Marinas y Costeras, (IIMyC, CONICET), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250. Mar del Plata, Argentina, CP 7600

[2]Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Ángel Gallardo 470, Buenos Aires, Argentina, C1405DJR

*Corresponding author.

Email: fmapelli@mdp.edu.ar

Abstract

We conducted a thorough survey of populations of *Ctenomys* within the distributional ranges of all species that currently represent the *Ctenomys mendocinus* species complex in Central Argentina. From the Atlantic Southeastern coast to the heights of the Los Andes mountain range we sampled individuals for all the previously recognized species within this species complex (*C. australis*, *C. azarae, C. porteousi, C. "chasiquensis"* and *C. mendocinus*). Sequences of different fragments of mitochondrial DNA were used to assess the phylogenetic relationships between individuals and to understand the processes responsible for the observed geographic distribution of genetic variation. The genetic differentiation among most of the sequences was moderated, although highly divergent haplotypes were recorded in some localities in the West of our study area, suggestive of the occurrence of a new so far undescribed species. The phylogenetic reconstructions suggested the presence of three major clades into *C. mendocinus* complex species that do not agree with the currently proposed taxonomy. Phylogeographic and demographic estimates indicated that the *C. mendocinus* species complex experienced a strong and recent process of population expansion. We propose that the pattern of distribution of haplotype diversity in the *C. mendocinus* species complex corresponds to a scenario of mild local differentiation with subsequent expansion to the current distribution ranges. Demographic changes through time suggest that glacial-interglacial cycles of Late Quaternary, and the consequent variation in the aridity of Central plains in Argentina, had strong effects on the evolution and cladogenesis of the *C*. *mendocinus* species complex.

Keywords: *Ctenomys mendocinus* species complex; Phylogeography; Central Argentina; Quaternary climate changes

Introduction

Delimiting species boundaries in recent radiations constitutes a staggering challenge (Shaffer and Thomson 2007; Sistrom et al. 2013). Subterranean rodents of the genus *Ctenomys* (tuco-tucos) represent a typical case of uncertainty on species boundaries (Parada et al. 2011; Fernández et al. 2012). The number of extant species into *Ctenomys* is highly debated nowadays, in part because a recent radiation affected the genus, and several interrelated species complexes evolved almost simultaneously (Lessa and Cook 1998; Castillo et al. 2005).

Tuco-tucos are distributed from Central Peru and South-eastern Brazil to the southernmost tip of Argentina, occurring in a wide variety of habitats from Atlantic coastal dunes to Andean valleys, around 5,000 meters above sea level (Cabrera 1961; Reig et al. 1990). Despite its moderate morphological and ecological diversity, *Ctenomys* is regarded as the most speciose genus of subterranean rodents, including 60-70 recognized species (Reig et al. 1990;

Slamovits et al. 2001; Castillo et al. 2005; Parada et al. 2011; Bidau 2015; Freitas 2016). The diversification started from the outset of the genus during the Late Pliocene, ~3 million ybp. (Reguero et al. 2007; Verzi et al. 2010), which suggests a fast cladogenetic process for this group (Lessa and Cook 1998; Castillo et al. 2005; Parada et al. 2011). Recent work achieved during the last two decades coming from biogeography, morphology, cytogenetics and phylogeography, helped to identify some species complex within the genus (Reig et al. 1990; D'Elía et al. 1999; Parada et al. 2011). Phylogenetic studies show a basal politomy among these species complexes, which presumably reflects the early and fast diversification within the genus (Lessa and Cook 1998; Castillo et al. 2005), and suggest that speciation in *Ctenomys* could have involved simultaneous diversification of lineages (Lessa and Cook 1998; Parada et al. 2011).

One of these lineages is the *Ctenomys mendocinus* species complex that includes at least four biological recognized species distributed in Central Argentina: *Ctenomys mendocinus*, *Ctenomys azarae*, *Ctenomys porteousi* and *Ctenomys australis*; together with one form of uncertain taxonomic status, *Ctenomys "chasiquensis"*. This latter form was mentioned by Contreras and Maceiras (1970) and then by Contreras (1973), but without proper taxonomic description, since there is no formal designation, diagnosis and description of a holotype. Afterwards, Mora et al. (2016) using a small fragment of mitochondrial DNA (mtDNA) found slight genetic differentiation between this entity and the other forms of this complex, but without consistent results to propose a new species. Thus, the name "*chasiquensis*" does not meet the criteria established by the International Code of Zoological Nomenclature (ICZN 1999: Art. 8) and is appropriately regarded as *nomen nudum*. Two additional geographically disconnected species are also included in the *Ctenomys mendocinus* species complex, *Ctenomys flamarioni,* which occurs along the coastal sand dunes in southeastern Brazil (De Freitas 1995); and *Ctenomys rionegrensis,* which occupies inland sand dunes between the Negro and Uruguay Rivers in Western Uruguay (D'Elia et al. 1998). However, the species from Argentina constitute a monophyletic clade relative to the other species of the *Ctenomys mendocinus* species complex (Parada et al. 2011).

Species richness within the genus *Ctenomys* is linked to an extraordinary amount of chromosomal variation both at the interspecific and intraspecific levels (Reig et al. 1990; Ortells 1995; Bidau et al. 1996; Mascheretti et al. 2000; Tomasco and Lessa 2007; Caraballo et al. 2012). In this regard, *Ctenomys* has been considered one of the best examples of rapid speciation mediated by chromosome rearrangements within mammals (Braggio et al. 1999). Although karyotypic variability seems to be the rule in this genus, the *C. mendocinus* species complex is characterized by the possession of a highly conserved karyotype morphology with the same 2n=48 (although limited polymorphism has been reported in some populations with 2n=46-48; see Massarini et al., 1998). In relation to this high karyotypic similarity, and considering that the role of chromosomal differences in promoting isolation has never been demonstrated in *Ctenomys* (Lanzone et al. 2007; Basheva et al. 2014), the chromosomal rearrangements seem not to play a central role in the evolution of the *C. mendocinus* complex species (Massarini et al. 1998).

Cytogenetic and morphological similarities, and mtDNA sequence matching, suggest a very recent divergence for lineages belonging to the *Ctenomys mendocinus* species complex (Massarini et al. 1991b; De Freitas 1994; Vitullo et al. 1988; D'Elía et al. 1999; Slamovits et al. 2001; Castillo et al. 2005; Parada et al. 2011). Massarini et al. (1998) have proposed that divergence within this group can be considered as an ongoing allopatric speciation process, involving gradual differentiation associated with local selective pressures. Low divergence among species of the group has also led to propose that this group is a single biological species with a wide geographical range in Central Argentina (Parada et al. 2011). According to this hypothesis, slight differentiation between lineages would be related only to local adaptive processes of the populations. These hypotheses are not mutually exclusive; while some lineages could have diverged enough to be considered as distinct biological species, others represent slightly differentiated populations of the same species.

The landscape inhabited by the *C. mendocinus* species complex has been severely impacted by paleoclimatic changes during the Late Quaternary, expanding or contracting their sandy habitats in response to arid pulses

(Quattrocchio et al. 2008). In this regard, the climatic evolution in Central Argentina was highly conditioned by the glacial-interglacial cycles of the Quaternary (Tonni et al. 1999). Fossil records indicate that climatic fluctuations alternated between arid and cold (glacial periods), and warm and humid conditions (interglacial periods). These fluctuations highly affected the landscape, especially in the eastern part of the distribution of the *C. mendocinus* species complex, where environments alternated between semi-arid and temperate prairies (Quattrocchio et al. 2008). Plant cover, a landscape variable that has been viewed to strongly impact the populations of the *C. mendocinus* species complex (Mapelli et al. 2012a, b; Mora et al. 2016), was severely impacted by these paleoclimatic changes (Prieto 2000).

In this work, we analyzed the genetic variation of mtDNA sequences of the Argentinian species belonging to the *C. mendocinus* species complex in order to understand the evolutionary processes underlying the cladogenesis of the group. Additionally, we analyzed the geographical distribution of genetic variation from a phylogeographic perspective, in order to understand how the evolutionary processes in this linage were modeled by the Late Quaternary paleoenvironmental changes. We hypothesize that the landscape evolution during the Late Quaternary in Central Argentina has strongly impacted the diversification patterns in the *C. mendocinus* species complex. Under this scenario, the increase of aridity during glacial pulses in Central Argentina would have increased the extension and availability of sandy habitats used most frequently by the tuco-tucos. We anticipate that this situation would result in an increase in population sizes and levels of gene flow among regions. On the contrary, when conditions of higher temperature and humidity restricted and fragmented habitats suitable for tuco-tucos, their populations would have diverged in association with differential local selection pressures and the random effect of genetic drift.

Materials and Methods

Study area and sample collections

The distributional range of the *Ctenomys mendocinus* species complex in Argentina extends from the Andean valleys to the Atlantic coastal sand dunes, occupying a wide variety of habitats occurring in arid, semi-arid and temperate climates in lowlands and mountainous regions. In the West and Central areas of the distribution, arid and semiarid environments inhabited by tuco-tucos constitute a more continuous habitat. The Andean valleys continue to the East into the ecoregion of Monte Desert, a semi-arid region of xerophytic woodland vegetation (Prieto 2000, Figure S1). Farther east of the Monte Desert appears the Espinal, a xeric region of low and spiny vegetation among the Monte desert and the Pampas Grassland (Figure S1). *C. mendocinus* inhabit Andean valleys and Monte Desert areas, whereas *C. azarae* occupy the Espinal, and also some regions of Monte Desert (Massarini et al. 1998; Rosi et al. 2002). The distributional ranges of these two species were defined in a very ambiguous mode and could be highly overlapping (Bidau 2015). To the East of Espinal, the rainfall increases and the landscape change gradually to humid temperate prairies, conforming the Pampas Grassland (Prieto 2000, Figure S1). On this latter landscape, the populations of the *C. mendocinus* species complex are present only in some isolated sandy regions. Thus, *C. porteousi* is restricted to a paleodune system originated into the deflation basins associated to “Encadenadas del Oeste” lagoons (Mapelli and Kittlein 2009; Mapelli et al. 2012a, b), whereas *C. “chasiquensis”* inhabit only some sandy paleo-drainage basins in the South of Pampas Grassland (Mora et al. 2016). In the same way, *C. australis* occupy only a narrow fringe of coastal sand dunes in the Southern Pampas Grasslands (Contreras and Reig 1965; Zenuto and Busch 1995; Mora et al. 2006; Mora and Mapelli 2010).

This study includes 97 specimens sampled throughout Central Argentina (Figure 1), including the type localities for all 5 taxa belonging to the *C. mendocinus* species complex. Distributional ranges and the extent of their respective habitats are best known for *C. australis*, *C. porteousi* and *C. “chasiquensis”* (Mora et al. 2006; Mapelli and Kittlein 2009; Mora et al. 2016, respectively). In these species, the studies did not show any relationship between genetic structure and geographical distance in their respective ranges. According to this background we included 7 individuals of *C. porteousi* sampled in three localities close to their type locality, 6 individuals of *C. australis* sampled in three localities along coastal sand dunes of Buenos Aires Province, and 18 individuals of *C. "chasiquensis"* sampled in four localities close to their type locality in the Chasicó Lake.

The distributional ranges of *C. mendocinus* and *C. azarae* are less known as well as ambiguously defined (Bidau 2015). Their described distributions are based only on studies of limited geographical extent that considered scarce sampling points (Massarini et al. 1991a, b; Rosi et al. 1992, 2002, 2005; D'Elía et al. 1999; Parada et al. 2011, 2012), but there is no assessment of the distribution of their populations or their habitats between or beyond these localities. In this survey we include new localities outside the distributional ranges previously delimited for each species, some of them at intermediate locations between the two species ranges. Therefore, only the specimens sampled in their type localities are referred as *C. mendocinus* (Cerro de la Gloria, Mendoza Province) or *C. azarae* (Gral. Acha, La Pampa Province). In this study, all other specimens are named based on the sampling localities without further *a priori* taxonomic inferences. Geographic coordinates and sample sizes for each locality are listed in Appendix 1. Additionally, we included 3 samples of populations assigned to *Ctenomys talarum* (Mora et al. 2013) that were used as outgroup in phylogenetic analyses.

Laboratory procedures

Tissue samples (first phalange of the toe) were preserved in 95% ethanol and the vouchers deposited at the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN). Total DNA was extracted following a protocol modified from Miller et al. (1988). Digestion with proteinase-K (10 mg/ml) was carried out in a buffer containing Tris-HCl 50 mM, EDTA pH 8 50 mM, NaCl 50 mM and 1% SDS. The DNA was precipitated with 5M NaCl.

We obtained three partial sequences of mtDNA. First, we sequenced two separate fragments of the control region (fragment A of 435 bp using the primers TucoPro [Tomasco and Lessa 2007] and TDKD [Kocher et al. 1989]), and fragment B of 464 bp using the primers MF272L and MF15876H [Tomasco and Lessa 2011]). The third fragment was obtained from the cytochrome b gene

(402 bp) using primers H15149 and L14724 (Kocher et al. 1989).

PCR amplification was carried out in a reaction volume of 20 µl using 30 ng of DNA, 0.4µM of each primer, 0.2mM of each dNTP, 1X Taq buffer (750mm Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 2.5-1.5 mM of $MgCl_2$ and 0.4 units of Taq polimerase (Fermentas). Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 3 blocks of 10 cycles of: 30 s denaturation at 94°C, 30 s annealing at 54°C-52°C for fragment A and cyt-b, and at 60°C-58°C for fragment B, and a 30 s extension at 72°C, with a final 5 min extension at 72°C. Negative control reactions were included in all PCR runs to detect contamination.

PCR products were examined in 1.2% agarose gels, and fragment size was contrasted against a commercial 100 bp Ladder (Invitrogen). PCR products were purified with Exonuclease I (which degrades residual primers), and a Shrimp Alkaline Phosphatase (to dephosphorylate unincorporated dNTPs). Purified PCR products were sequenced in an ABI3100 sequencer (MACROGEN Inc., Korea), with the primers used during the PCR amplification.

Data analyses

Electropherograms were scored and analyzed using Chromas 2.01 (Technelysium,Helensvale, Qld, Australia) and aligned using CLUSTAL X (Thompson et al. 1997). All sequences used in the analyses were obtained in this study. GenBank accession numbers were included in the Appendix 1.

Phylogenetic inference was performed using algorithms from maximum likelihood (ML), Bayesian (B) and Neighbor Joining (NJ). The combined data set was tested for incongruence with the partition homogeneity test (Farris et al. 1995), as implemented in PAUP* (Swofford 2002). The result of this test showed that the sequence data sets for the three loci were congruent ($P = 0.30$) and could therefore be combined. Thus, a concatenated fragment of mitochondrial DNA adding 1302 bp was used. Three sequences of *Ctenomys talarum* were used as outgroup.

Maximum Likelihood phylogenetic inference was performed using the program PhyML 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010). This program performs simultaneous nearest neighbor's interchanges in order to improve a reasonable starting tree topology. PhyML was run under the best nucleotide substitution model, previously inferred with jModelTest (Posada 2008). Both, the relationship between transitions and transversions and the distribution of the gamma parameter, were estimated empirically. Consistency of internal branches and nodes was evaluated with the standard bootstrap method (sampling with replacement, using 1,000 bootstrap replicates).

The program BEAST 1.5.4 was used to infer the phylogenetic tree topology using the Bayesian Markov chain Monte Carlo method (Drummond and Rambaut 2007). Each chain consisted of 1 x 10^7 iterations with parameters sampled every 1,000 steps, assuming an uncorrelated relaxed lognormal molecular clock with uniform priors and the nucleotide substitution model selected by jModelTest (Posada 2008). The first 8,000 samples were considered as burn-in and discarded. A consensus phylogeny was inferred from the last 2,000 samples of the chain. Four independent chains were run to assess convergence of the estimation procedure using the program Tracer 1.4.8 (Rambaut et al. 2014).

Phylogenetic inference with Neighbor Joining (NJ) was performed using Mega 6.06 (Tamura et al. 2013). As before, the relationship between transitions and transversions and the distribution of the gamma parameter were estimated empirically. Consistency of internal branches and nodes was evaluated with the standard bootstrap method (sampling with replacement, using 1,000 bootstrap replicates). The topology of the phylogenetic tree was edited with the program FigTree 1.4 (Rambaut 2009).

The relationship among haplotypes was also analyzed constructing a haplotype network with Median-joining methods (Bandelt et al. 1999) as implemented in Network 4.5.1 (http://www.fluxus-emgineering.com). Uncorrected genetic distances between sequence groups reported in results were computed using Mega 6.06 (Tamura et al. 2013).

The historical demography was inferred using different complementary approaches. First, we tested for sudden demographic expansion using mismatch distribution analyses. We employed the sum of squared deviations (SSD) statistic and the raggedness index (Rg) defined by Harpending (1994) to test the goodness of fit of the observed mismatch distribution to that expected under the sudden expansion model. Estimation and testing were done by bootstrap resampling (10,000 replicates) using ARLEQUIN 3.0 (Excoffier et al. 2005). Additionally, to estimate demographic changes through time we constructed Bayesian skyline plots (BSP) as implemented in BEAST 1.4.8 (Drummond and Rambaut 2007). This approach incorporates the uncertainty in the genealogy by using MCMC integration under a coalescent model, where the timing of divergence events provides information about changes in effective population size through time. Demographic model was chosen based on the Bayesian factor (BF) scores, calculated by estimating of the marginal likelihood of different models using both path sampling and stepping stone methods as proposed by Baele et al. (2012). MCMC tests were run for 1×10^8 iterations and sampled every 5,000 steps under a relaxed lognormal molecular clock with uniformly distributed priors. The first 10% iterations were discarded as a burn-in period. To assess the robustness of parameter estimates, independent chains were run with identical settings. Log-files were analyzed in Tracer 1.4.8 (Drummond and Rambaut 2007), and effective sample sizes were used to evaluate MCMC convergence within chains. To convert coalescence times to years from the substitutions per site estimated by BEAST, we used a mutation rate of 4.42% Myr $^{-1}$ estimated for the mitochondrial control region of the genus *Ctenomys* by Mora et al. (2013). Additionally, because molecular clock estimates are often dubious, we applied different mutation rates, using an interval between 1.7 and 10% per million years (Myr), which conservatively bracket the range of mtDNA mutation rates proposed for other Ctenomyids and Octodontids (Gallardo et al. 2013; Roratto et al. 2015; Rivera et al. 2016).

Finally, a detailed pattern of spatial genetic structure across the complete sampled area were visualized using the “genetic landscape shape” (GLS) interpolation procedure as implemented in Alleles in Space (AIS) software (Miller 2005). This procedure employs an inverse distance weighted interpolation method to generate surface plots of genetic variation across landscape. Midpoints of edges derived of Delaunay triangulation and residual genetic distances between points were used for the analyses. To visually display the AIS results in a geographical context, we used DIVA-GIS 7.5.0 and plotted them on a map of Central Argentina.

In spite of some taxa of *C. mendocinus* complex species are recognized as valid species, both for the demographic and GLS analyzes we pooled all sequences assigned to this complex species in the same database. We based this decision in the low divergence observed among *C. mendocinus* lineages and the absence of reciprocal monophyly among recognized biological species (see results).

Results

For the D-loop mitochondrial region, 101 polymorphic positions were recorded (51 in fragment A and 50 in fragment B); while in the cytochrome b we found 52 polymorphic sites. Thus, the concatenated sequences yielded 1303 bp with 153 polymorphic sites, including 8 indels located in the fragment B of the D-Loop sequences, defining a total of 86 haplotypes.

Phylogenetic relationships among haplotypes are presented in Figures 2 and 3. First, a profound divergence between the sequences of individuals from the sampling sites of Las Leñas, Arenales and Quijadas was observed relative to the other haplotypes. Genetic distances between individuals belonging to these latter localities and the other samples assigned to the *C. mendocinus* clade (2, 2.5 and 1.8%, respectively, see Table 1) were equivalent to the distance observed between *C. mendocinus* clade and the outgroup (2.8%), so the tuco-tucos inhabiting those three localities probably do not belong to the *C. mendocinus* species complex. Moreover, the divergence observed among the three locations was also very high (1.9-2.9%) and similar to that observed in the interspecific comparisons with *C. talarum* (2.5-3.4 %, see Table 1), suggesting that individuals of Arenales, Las Leñas and Quijadas could be independent taxa.

Three clusters of haplotypes with relatively good support were observed within the *C. mendocinus* species complex. One of the clades has a wide geographic distribution covering the entire Northern distributional area of the *C. mendocinus* species complex from Andean foothills to the Atlantic coastal dunes (Clade N, Figures 2, 3 and 4). This clade includes the type localities of *C. mendocinus*, *C. porteousi*, *C. australis* and *C. “chasiquensis”*. All individuals collected in North-Central distributional ranges belong to this clade, as well as all individuals collected within the distributional ranges assigned to *C. porteousi* and *C. australis*. The clade also contains the samples from Central part of distributional ranges of species complex, areas traditionally assigned to *C. azarae*; but does not include samples from the type locality of this last species. In comparison to Clade N, the second clade (Clade C, Figures 2, 3 and 4) has a limited geographic distribution, restricted to some Central locations. This clade contains six individuals collected within the distributional range of *C. “chasiquensis”*, one specimen collected in Chacharramendi, and one individual collected in Río Desaguadero, both populations in the Center of distributional ranges of species complex (see Figure 1). Finally, the third clade (Clade SW, Figures 2, 3 and 4) includes the type locality of *C. azarae* (Gral. Acha), and was present fundamentally in Southwestern part of distributional ranges of species complex, areas traditionally assigned to *C. azarae* or *C. mendocinus.* Estimates of genetic diversity by clade are shown in Appendix 2.

The relationship between these three clades was not clear. Some phylogenetic methods suggested a higher relationship among clade SW and clade C, whereas other showed that clade C is more related to Clade N than Clade SW. Moreover, the node that sustains this separation has low bootstrap support by both ML and NJ methods and low posterior probability by the Bayesian method (Figure 2). Also, the genetic distances between these three major clades were low to moderate (Table 1).

In most localities, the haplotypes belong to Clade N or to Clade SW. The haplotypes characterizing Clade C are present in only four locations, and in all cases co-occurring with haplotypes that belong to one of the other two clades. Only two sampling localities had haplotypes belonging to clades N and SW: Villa Mercedes and the depression of Chasicó Lake; in both cases the proportion belonging to clade N was predominant. Within each clade, relationships between haplotypes were unclear, had a reticulated appearance in the haplotype network and were recovered mostly as polytomies and/or having low support of the nodes in the tree in all phylogenetic analyses.

As for the phylogenetic relationships among recognized species, only *C. australis* was recovered as a monophyletic group, although it did not show reciprocal monophyly with any of the other taxa. *C. porteousi* was recovered as polyphyletic, although with all individuals within the same main clade (Clade N). This latter species were divided in two mitochondrial haplogroups: one is closely related to *C. australis* and part of the samples within the range of *C. "chasiquensis"*; while the other had a closer relationship with tuco-tucos from Central-northern part of distributional ranges of the species complex. Tuco-tucos sampled in the *C. azarae* type locality (Gral. Acha) were included in the SW clade and showed a close relationship with geographically closer populations which have not been assigned to any recognized taxa (only tuco-tucos from Cerro Centinela were assigned to *C. azarae* in previous studies, see Massarini et al. [1998]). However, the rest of the localities in Central-northern part of distributional ranges of species complex, traditionally assigned to *C. azarae* showed a closer relationship with Clade N. Tuco-tucos from the type locality of *C. mendocinus* (Cerro de la Gloria) showed a closer relationship, although with low bootstrap support, with tuco-tucos from the nearby localities Uspallata and Ñacunan, also assigned to that species. It should be noted, however, other localities within the proposed distribution of this species in previous works showed haplotypes characteristic of clades C and SW. Finally, *C. "chasiquensis"* was also recovered as polyphyletic: most individuals sampled within its range were grouped within the clade N (65%) or clade C (30%), and only one individual showed a closer relationship with clade SW.

Mismatch distribution of pairwise differences among haplotypes was markedly unimodal suggesting a recent demographic expansion of the *C. mendocinus* species complex (Figure 5A). Both SSD (SSD=0.002, P=0.57) and Harpending's raggedness index (Rg=0.001, P=0.99) indicated that the observed mismatch distribution did not differ significantly from the distribution expected under population expansion.

When different demographic models were compared the non-parametric skyline model significantly outperformed other parametric ones (constant population size, exponential growth and expansion growth, BF > 20). The demographic scenario inferred from the BSP analysis was concordant with a population expansion hypothesis (Figure 5B). The effective population size showed a sustained increment starting about 70,000 ybp under a mutation rate of 4.42% Myr^{-1} (between 200,000 and 40,000 ybp using 1.7 and 10% My^{-1} respectively). During recent times (middle and Late Holocene), the BSP reconstruction suggested also a small reduction in effective population size.

Finally, the two-dimensional GLS plot revealed that the region containing the greatest genetic distances between sampling localities was located in the Central and Western areas of the distributional range of the *C. mendocinus* species complex (Figure 6). Conversely, the relatively low mitochondrial sequence variability was observed among populations found in some peripheral areas of the distribution of the group. Particularly, the Northeast and Southwest limits of distribution of the *C. mendocinus* and the distributional area of *C. australis* showed the lowest genetic variation among sampling localities. Changes of the model parameters and grid cell size did not affect the overall pattern of GLS results.

Discussion

In this paper we analyzed the phylogenetic relationships and levels of genetic variation in the mitochondrial DNA of tuco-tucos distributed across Central Argentina, traditionally grouped in the *C. mendocinus* species complex and assigned to *C. mendocinus*, *C. azarae*, *C. porteousi*, *C. australis* or *C. "chasiquensis"*. These taxa have shown a close phylogenetic relationship in previous analysis (Massarini et al. 1991a; Vitullo et al. 1988; Parada et al. 2011) and have been suggested to be a single widespread species or a group of populations experiencing an ongoing speciation process (Massarini et al. 1998; Parada et al. 2011).

Phylogenetic relationship among tuco-tucos of the *C. mendocinus* species complex.

Our results clearly indicate that the tuco-tucos from Central Andes and from the locality of Quijadas do not belong to the *C. mendocinus* species complex. The divergence between these samples and the remaining localities is similar to that observed with *C. talarum* (a species pertaining to other phylogenetic group); therefore, these tuco-tucos most likely constitute a different species. Similarly, the divergence between samples from Arenales, Las Leñas and Quijadas is equivalent to that observed between them and the *C. mendocinus* species complex or *C. talarum*, so most likely in each of these locations a different species of tuco-tuco occurs. Sequences from Arenales show a close relationship with sequences from Tupungato (uncorrected genetic distances between 1.4-1.7 %) published by Parada et al. (2011), who also suggested that these tuco-tucos belong to a distinct species from *C. mendocinus*.

Within the *C. mendocinus* species complex, our results are broadly consistent with the previous evidence where very little divergence among lineages was observed (Massarini et al. 1991b; Vitullo et al. 1988; Castillo et al. 2005; Parada et al. 2011). Besides, the geographic distribution of major defined genetic groups (clades N, SW and C) does not match the range assumed for recognized species in previous studies (Contreras and Reig 1965; Massarini et al. 1991a; Apfelbaum et al. 1991; Bidau 2015). Thus, the clades defined in our analysis are in disagreement with the taxonomy currently proposed for the *C. mendocinus* species complex. In this paper we do not attempt to sustain any alternative taxonomic proposal, but only to confront our results against previous evidence, trying to understand the processes that led to the observed distribution of genetic variation.

The three major clades recovered in our analysis show a strong geographic component. The SW clade is distributed primarily in the Southwestern distributional area; the C clade is restricted to some central localities and, the N clade covers all the Northeastern fringe of the range of the species complex, occupying regions traditionally assigned to *C. mendocinus*, *C. azarae* (without considering its type locality), *C. porteousi*, *C. australis* and *C. “chasiquensis”*. The divergence between sequences of the three clades recovered in our analysis was in the order of 1.2% to 1.5%, a similar value to the average of intraspecific divergence between tuco-tucos belonging to other different phylogenetic groups observed by Parada et al. (2011) using mitochondrial DNA. Moreover, Massarini et al. (1998) and Braggio et al. (1999) analyzed the variation in chromosome polymorphisms of tuco-tucos located at Central Argentina and found very little variation among localities that here we report belonging to different clades. This low divergence, along with the co-occurrence of haplotypes of different clades in the same locality, leads us to suggest that these three clades neither represent nor characterize distinctive taxonomic units.

The lineages that have had enough time to diverge often reach a set of features, including reproductive isolation, fixation of apomorphies and gene tree monophyly, which allow a robust definition of the limits of the species. However, very young lineages that are in the early stages of speciation often lack from some of these features, appearing as particularly problematic for systematic delineation (Shaffer and Thomson 2007). Considering reciprocal monophyly as a necessary condition for the delimitation of species, our data indicate that *C. porteousi*, *C. australis*, *C. azarae* and *C. “chasiquensis”* should be synonymized to *C. mendocinus*. However, monophyly is a condition that takes a considerable amount of time to be established, and is therefore very rare among taxa of recent divergence (Hudson and Coyne 2002). Retention of ancestral polymorphisms and lineage sorting increase the time necessary to achieve reciprocal monophyly (Maddison 1997; Rosenberg 2002). Thus, for lineages that diverged recently, it is highly probable that the gene tree does not stand for the species tree (Sites and Crandall 1997; Maddison and Knowles 2006; Kubatko and Degnan 2007). Under this scenario, gene trees provide a partial and incomplete evidence to establish more robust systematic conclusions. The results of this study indicate that divergence among taxa of *C. mendocinus* complex species is too recent to be understood only with the evidence provided for our database (three fragments of mitochondrial genome). A more comprehensive approach, including assessment of morphological, karyological and ecological variability, is necessary to determine the taxonomic status of the tuco-tucos within the *C. mendocinus* species complex. Taking into account the wide geographical and environmental distribution of the *C. mendocinus* species complex, it is likely that a wide range of evolutionary conditions molded the current pattern of variation, generating from slightly differentiated populations to incipient species.

The phylogeny of tuco-tucos permits to identify several species complexes within *Ctenomys* (Reig et al. 1990; D'Elía et al. 1999). Molecular data show low diversity within these species complexes and suggest recent and simultaneous radiations (Lessa and Cook 1998; Castillo et al. 2005; Parada et al. 2011). Similar to this study, low interspecific divergence characterize other species complexes within *Ctenomys* genus (Caraballo et al. 2012; Fernández et al. 2012). In this context, we suggest that species complexes of *Ctenomys* could be currently undergoing speciation processes, so that to delimit species within each complex constitutes a daunting challenge.

On the other hand, tuco-tucos are highly specialist in habitat use and suffer strongly the effects of anthropogenic landscape changes (Mapelli and Kittlein 2009; Mapelli et al. 2012b; Mora et al. 2016). This situation, added to the fact that many species of *Ctenomys* have very restricted distributional ranges, determines that several species of tuco-tucos are considered threatened. The existence of cryptic diversity and species complexes within *Ctenomys* greatly obscures the determination of their conservation status. Under some taxonomic hypotheses, endemic species highly threatened could be considered populations of the same species with a greater distributional area and, consequently, better conservation status (this would be the case of *C. australis* and *C. porteousi* if they are synonymized to *C. mendocinus*). On the contrary, alternative taxonomic hypothesis would imply that some sufficiently differentiated populations will be considered species. These species could have a greater endemism and probably face onto seriously differ conservation scenarios. Thus, the resolution of alpha taxonomy of *Ctenomys* is urgently necessary to valid the conservation efforts of tuco-tucos populations.

Phylogeographical pattern of the *C. mendocinus* species complex

The almost allopatric geographical distribution of the main clades recovered in our phylogenetic analysis (mainly clades N and SW) points out to different histories and limited gene flow among the regions they occupy. The presence of haplotypes belonging to more than one clade in some localities may result from the retention of ancestral polymorphisms, or to recent gene flow due to secondary contact between previously distinct populations. If gene flow were responsible for this pattern we should assume a scenario of environmental connectivity that has allowed the exchange of individuals between sites. Thus, is expected that shared haplotypes due to gene flow should be evident in most sampling locations, so a higher number of these localities would show haplotypes belonging to more than one clade. Since only four localities had haplotypes belonging to more than one clade, retention of ancestral polymorphisms in these sites appears to be the most parsimonious explanation for the observed pattern.

Alternatively, most localities were represented by only one phylogenetic clade, suggesting strong genetic drift in these sites, which could have been brought by both fluctuating population sizes as well as successive founder events that decreased genetic diversity. Under a scenario of fluctuating changes in population sizes, genetic drift acts reducing variability within populations, but this should have to be an independent and random process for each population. Therefore, is not expected that haplotype frequencies across populations were geographically correlated (Mapelli et al. 2012a; Mora et al. 2016).

Accordingly, the strong geographic component regarding the current distribution of these clades would be most parsimoniously explained by a process of population expansion, associated with strong founder events. Consequently, the demographic analyses indicated strong signals of recent population expansion for the *C. mendocinus* species complex. In this context, the distribution of genetic variation in the *C. mendocinus* species complex seems to be due to a combination of processes involving demographic stability in some areas and recent colonization of a large portion of its current distribution in other regions. According with this scenario, GLS results indicated geographic areas with higher genetic diversity, which could be assumed as areas with higher demographic stability, and peripheral zones that presented a reduced genetic diversity, similar to that expected in areas recently colonized. In sum, we inferred a recent expansion of the *C. mendocinus* species complex from West and Central to peripheral areas in the Southwestern, Southeastern and Northeastern limits of their current distribution.

Late Quaternary paleoclimatic changes and the evolution of the *C. mendocinus* species complex

It is well known that the degree of plant cover significantly affects various population processes in tuco-tucos belonging to the *C. mendocinus* species complex (Mapelli and Kittlein 2009; Mapelli et al. 2012b). A clear example of this situation was found in *C. porteousi*, where patch occupancy was strongly influenced by plant cover (less vegetated patches were more likely to be occupied, Mapelli and Kittlein 2009). Furthermore, plant cover was also one of the environmental variables with greater effect on the distribution of *C. australis* (Mora and Mapelli 2010). In this sense, studies of molecular ecology in these species showed that the degree of population structure and gene flow were strongly affected by habitat fragmentation and connectivity between less vegetated patches (Mora et al. 2010; Mapelli et al. 2012b). This negative effect of plant cover on the populations of these tuco-tucos was also evident in the reconstruction of their historical demography. Both in *C. porteousi* (Mapelli et al. 2012a) as in *C. “chasiquensis”* (Mora et al. 2016) a marked process of population decline was inferred from the Pleistocene-Holocene boundary, a period where their distributional ranges experienced a sharp decline in aridity (Borromei 1995; Grill 1997; Quattrocchio et al. 1998, 2008).

The Pleistocene glaciations had strong effects on climate and landscape in Central Argentina (Rabassa et al. 2005). During glacial periods, colder and drier conditions than present were reported, whereas the interglacial periods were characterized by an increase in temperature and humidity (Quattrocchio et al. 2008). These environmental changes strongly affected the distribution of biota in the area, producing an increase of Brazilian species during the warm-wet periods and Patagonian-Monte elements during cold-arid pulses (Tonni et al. 1999). Given the strong influence that plant cover has on the dynamics and population structure of these tuco-tucos (Mora and Mapelli 2010; Mora et al. 2010, 2016; Mapelli et al. 2012a, b), it is expected that the cycles of aridity in Central Argentina have strongly affected the biogeographic history of the *C. mendocinus* species complex. According to the scenario of landscape changes proposed for the Pleistocene–Holocene in the central area of Argentina (Rabassa et al. 2005) an expansion of the distributional range of the *C. mendocinus* species complex during glacial pulses, and a retraction during interglacial periods, is expected. These processes would have produced a distribution of genetic variation similar to that observed in our data: localities where populations of tuco-tucos were retracted during interglacial periods could retain a high genetic diversity, whereas the recently colonized areas could reflect strong founder events.

The strongest demographic change inferred for the *C. mendocinus* species complex implied a marked increase in population size started about 70,000 ybp. Paleoclimate estimates indicate that this period was the coldest and driest time for the last glacial-interglacial cycle (Iriondo and Kröhling 1995; Iriondo 2010). These climatic conditions generated an increase in the extension of arid environments and may have permitted a wider and more continuous distribution of tuco-tucos of *C. mendocinus* species complex throughout the Pampas in Central Argentina.

As was suggested previously, wetter-warmer periods alternated with colder-drier times in the Late Pleistocene/Holocene (Iriondo 1994). Thereby, arid environments could retract during humid periods and could expand during arid pulses. It is expected that connectivity between populations has increased during the process of habitat expansion generating an increase in the levels of gene flow among populations that had somewhat differentiated. Moreover, during the process of habitat retraction, the combined effects of natural selection and genetic drift would favor the divergence between different lineages. Thus, the divergence among lineages of the *C. mendocinus* species complex may have taken place during humid intervals, when climate conditions resulted in environmental discontinuities. This hypothesis suggests that evolution and differentiation in the *C. mendocinus* species complex was conditioned by the magnitude of gene flow during periods of habitat connectivity and the magnitude of genetic drift during periods of habitat fragmentation.

A similar pattern of lineage diversification associated to the landscape evolution in the Late Pleistocene/Holocene seems to have occurred in the sand lizards of the *Liolaemus multimaculatus* species complex (Albino 2005). It is remarkable that, as tuco-tucos of the *C. mendocinus* species complex, *L. multumaculatus* species complex are associated to sandy and poor vegetated habitats (Mapelli and Kittlein 2009; Stellatelli et al. 2014). Thus, the Late Quaternary paleoclimatic changes associated to glacial-interglacial cycles seems to have affected the evolution, if not of all, at least of those psamophitic species in Central Argentina.

Acknowledgements

We are grateful to all the members of the GECoBi, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” and the Laboratorio de Ecofisiología, Universidad Nacional de Mar del Plata, for their invaluable support and advice. We are grateful to Dra. Agustina Ojeda by offering us samples for some localities of Mendoza Province. To these people and institutions, we express our deep gratitude. This work was supported by the National Agency for the Promotion of Scientific and Technological Research (PICT 2010-1551), the National Council of Scientific and Technical Researches (PIP-2011-2013-1380), and the National University of Mar del Plata (15 EXA598/12).

References

Albino, A.M. 2005. A late Quaternary lizard assemblage from the southern Pampean Region of Argentina. J Vertebr Paleontol 25: 185-191.

Apfelbaum, L.I., Massarini, A.I., Daleffe, L.E., Reig, O.A. 1991. Genetic variability in the subterranean rodents *Ctenomys australis* and *Ctenomys porteousi* (Rodentia: Octodontidae). Biochem Syst Ecol 19: 467-476.

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M. A., Alekseyenko, A. V. 2012. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Mol Biol Evol 29: 2157-2167.

Bandelt, H.J., Forster, P., Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16: 37-48.

Basheva, E.A., Torgasheva, A.A., Fernandez, M.J.G., Boston, E., Mirol, P., Borodin, P.M. 2014. Chromosome synapsis and recombination in simple and complex chromosomal heterozygotes of tuco-tuco (*Ctenomys talarum*: Rodentia: Ctenomyidae). Chromosome research 22: 351-363.

Bidau, C.J., Gimenez, M.D., Contreras, J.R. 1996. — Especiación cromosómica y la conservación de la variabilidad genética: El caso del genero *Ctenomys* (Rodentia, Caviomorpha, Ctenomyidae). Mendeliana 12: 25-37.

Bidau, C.J. 2015. Family Ctenomyidae Lesson, 1842. . In Patton, J.L., Pardiñas, U.F., D'Elía, G. (eds.). Mammals of South America, Volume 2: Rodents, pages 818-877. University of Chicago Press.

Borromei, A.M. 1995. Palinologa, estratigrafa y paleoambientes del Pleistoceno Tardo-Holoceno en el Valle del Río Sauce Grande, Provincia de Buenos Aires, Argentina. Polen 7: 19-31.

Braggio, E., Gimenez, M., Contreras, J., Justo, E., Bidau, C.J. 1999. Karyotypic variation in populations of *Ctenomys* (Rodentia, Ctenomyidae) from La Pampa Province (Argentina). Caryologia 52: 131-140.

Cabrera, A. 1961. Catálogo de los mamíferos de América del Sur. Revista del

Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (Ciencias Zoológicas) 4: 309-732.

Caraballo, D.A., Abruzzese, G.A., Rossi, M.S. 2012. Diversity of tuco-tucos (*Ctenomys*, Rodentia) in the Northeastern wetlands from Argentina: mitochondrial phylogeny and chromosomal evolution. Genetica 140:125-136.

Castillo, A.H., Cortinas, M.N., Lessa, E.P. 2005. Rapid diversification of South American tuco-tucos (*Ctenomys*; Rodentia, Ctenomyidae): contrasting mitochondrial and nuclear intron sequences. J Mammal 86: 170-179.

Contreras, J.R. 1973. La mastofauna de la zona de la Laguna Chasicó, Provincia de Buenos Aires. Physis 32: 215-219.

Contreras, J.R., Maceiras, A.J. 1970. Relaciones entre tucu-tucos y los procesos del suelo en la región semiárida del sudoeste bonaerense. Agro 12: 3-17.

Contreras, J.R., Reig, O.A. 1965. Datos sobre la distribución del género *Ctenomys* (Rodentia: Octodontidae) en la zona costera de la provincia de Buenos Aires comprendida entre Necochea y Bahía Blanca. Physis 25: 169-186.

D'Elía, G., Lessa, E., Cook, J. 1998. Geographic structure, gene flow, and maintenance of melanism in *Ctenomys rionegrensis* (Rodentia: Octodontidae). Z Saugetierk 63: 285-296.

D'Elía, G., Lessa, E., Cook, J. 1999. Molecular phylogeny of Tuco-Tucos, genus *Ctenomys* (Rodentia: Octodontidae): Evaluation of the mendocinus species group and the evolution of asymmetric sperm. J Mamm Evol 6: 19-38.

De Freitas, T.R.O. 1994. Geographical variation of heterochromatin in *Ctenomys flamarioni* (Rodentia-Octodontidae) and its cytogenetic relationships with other species of the genus. Cytogenet Cell Genet 67:193–198.

De Freitas, T.R.O. 1995. Geographic distribution and conservation of four species of the genus *Ctenomys* in southern Brazil. Stud Neotrop Fauna E 30: 53-59.

Drummond, A., Rambaut, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214.

Excoffier, L., Laval, G., Schneider, S. 2005. ARLEQUIN ver. 3.0: an integrated software packaged for population genetics data analysis. Evol Bioinform 1: 47-50.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C. 1995. Testing significance of incongruence. Cladistics 10:315-319.

Fernández, M.J.G., Gaggiotti, O.E., Mirol, P. 2012. The evolution of a highly speciose group in a changing environment: are we witnessing speciation in the Iberá wetlands? Mol Ecol 21: 3266-3282.

Freitas, T.R.O. 2016. Family Ctenomyidae (Tuco-tucos). Pp 498-534 in Wilson, D.E., Lacher, T.E., Mittermeier, R.A. (eds.). 2016. Handbook of the mammals of the world. Vol. 6. Lagomorphs and Rodents I. Lynx Edicions, Barcelona.

Gallardo, M.H., Suárez-Villota, E.Y., Nuñez, J.J., Vargas, R.A., Haro, R., Köhler, N. 2013. Phylogenetic analysis and phylogeography of the tetraploid rodent Tympanoctomys barrerae (Octodontidae): insights on its origin and the impact of Quaternary climate changes on population dynamics. Biol J Linn 108: 453-469.

Grill, S. 1997. Palinología de un perfil cuaternario en el Valle de Napostá Grande: Buenos Aires, Argentina. Polen 8: 24-42.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59: 307-321.

Guindon, S., Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696-704.

Harpending, R.C. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Hum Biol 66: 591-600.

Hudson, R.R., Coyne, J.A. 2002. Mathematical consequences of the genealogical species concept. Evolution 56: 1557-1565.

Iriondo, M. 1994. Los climas cuaternarios de la región pampeana. Com. Mus. Cs. Naturales Florentino Ameghino 4: 1-48.

Iriondo, M.H. 2010. Geología del cuaternario en Argentina. Moglia Press. Corrientes, Argentina, pp 437.

Iriondo, M., Kröhling, D. 1995. El sistema eólico pampeano. Com. Mus. Cs. Naturales Florentino Ameghino 5: 1-79.

Kocher, T., Thomas, W., Meyer, A., Edwards, S., Paabo, S., Villablanca, F., Wilson, A. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. P Natl Acad Sci 86: 6196-6200.

Kubatko, L.S., Degnan, J.H. 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. Syst Biol 56: 17-24.

Lanzone, C., Gimenez, M., Santos, J.L., Bidau C. J. 2007. Meiotic effects of Robertsonian translocations in tuco-tucos of the *Ctenomys perrensi* superspecies (Rodentia: Ctenomyidae). Caryolgia 60: 233-244.

Lessa, E., Cook, J. 1998. The molecular phylogenetics of tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae) suggests an early burst of speciation. Mol Phylogenet Evol 9: 88-99.

Maddison, W.P. 1997. Gene trees in species trees. Syst Biol 46: 523-536.

Maddison, W.P., Knowles, L.L. 2006. Inferring phylogeny despite incomplete lineage sorting. Syst Biol 55: 21-30.

Mapelli, F.J., Kittlein, M.J. 2009. Influence of patch and landscape characteristics on the distribution of the subterranean rodent *Ctenomys porteousi*. Landscape Ecol 24: 723-733.

Mapelli, F.J., Mora, M.S., Mirol, P.M., Kittlein, M.J. 2012a. Effects of Quaternary climatic changes on the phylogeography and historical demography of the subterranean rodent *Ctenomys porteousi*. J Zool 286: 48-57.

Mapelli, F.J., Mora, M.S., Mirol, P.M., Kittlein, M.J. 2012b. Population structure and landscape genetics in the endangered subterranean rodent *Ctenomys porteousi*. Conserv Genet 13: 165-181.

Mascheretti, S., Mirol, P.M., Gimenez, M.D., Contreras, J.R., Bidau, C.J., Searle, J.B. 2000. Phylogenetics of the speciose and chromosomally variable rodent genus *Ctenomys* (Ctenomyidae, Octodontoidea), based on mitochondrial cytochrome b sequences. Biol. J. Linn. Soc. 70, 361–376

Massarini, A.I., Barros, M., Ortells, M., Reig, O.A. 1991a. Chromosomal polymorphism and small karyotypic differentiation in a group of *Ctenomys species* from Central Argentina (Rodentia: Octodontidae). Genetica 83: 131-144.

Massarini, A.I., Barros, M.A., Roig, V.G., Reig, O.A. 1991b. Banded karyotypes of *Ctenomys mendocinus* (Rodentia: Octodontidae) from Mendoza, Argentina. J Mammal 72: 194-198.

Massarini, A.I., Dyzenchauz, F.J., Tiranti, S.I. 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.

Miller, M.P. 2005. Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. J Hered 96: 722-724.

Miller, S., Dykes, D., Polesky, H. 1988. A simple salting procedure for extracting DNA from nucleated cells. Nucleic Acids Res 16: 1215.

Mora, M.S., Cutrera, A.P., Lessa, E.P., Vassallo, A.I., D’anatro, A., Mapelli, F.J. 2013. Phylogeography and population genetic structure of the Talas tuco-tuco (*Ctenomys talarum*): integrating demographic and habitat histories. J Mammal 94: 459-476.

Mora, M.S., Lessa, E.P., Kittlein, M.J., Vassallo, A.I. 2006. Phylogeography of the subterranean rodent *Ctenomys australis* in sand-dune habitats: evidence of population expansion. J Mammal 87: 1192-1203.

Mora, M.S., Mapelli, F.J. 2010. Conservación en médanos: Fragmentación del hábitat y dinámica poblacional del tuco-tuco de las dunas. In Isla, F.I., Lasta, C.A. Manual de manejo de barreras medanosas de la Provincia de Buenos Aires, pp 161–181. Universidad de Mar del Plata ed. Mar del Plata, Argentina

Mora, M.S., Mapelli, F.J., Gaggiotti, O., Kittlein, M.J., Lessa, E.P. 2010. Dispersal and population structure at different spatial scales in the subterranean rodent *Ctenomys australis*. BMC genetics 11: 9.

Mora, M.S., Mapelli, F.J., López, A., Gómez Fernández, M.J., Mirol, P.M., Kittlein, M.J. 2016. Population genetic structure and historical dispersal patterns in the subterranean rodent *Ctenomys “chasiquensis”* from the southeastern Pampas region, Argentina. Mamm Biol 81: 314-325.

Ortells, M.O. 1995. — Phylogenetic analysis of G-banded karyotypes among the South American rodents of the genus *Ctenomys* (Caviomorpha: Octodontidae), with special reference to chromosomal evolution and speciation. Biol. J. Linn. Soc., 54: 43-70.

Parada, A., D’Elía, G., Bidau, C.J., Lessa, E.P. 2011. Species groups and the evolutionary diversification of tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). J Mammal 92: 671-682.

Parada, A., Ojeda, A., Tabeni, S., D'Elía, G. 2012. The population of *Ctenomys* from the Ñacuñán Biosphere Reserve (Mendoza, Argentina) belongs to *Ctenomys mendocinus* Philippi, 1869 (Rodentia: Ctenomyidae): molecular and karyotypic evidence. Zootaxa 3402: 61-68.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol Biol Evol 25: 1253-1256.

Prieto, A.R. 2000. Vegetational history of the Late glacial–Holocene transition in the grasslands of eastern Argentina. Palaeogeogr Palaeoc 157: 167-188.

Quattrocchio, M.E., Grill, S., Zavala, C.A. 1998. Chronostratigraphic and palynozone chronosequences charts of Napostá Grande creek, Southwestern Buenos Aires Province, Argentina. Quat S Am A 11: 111-133.

Quattrocchio, M.E., Borromei, A.M., Deschamps, C.M., Grill, S., Zavala, C.A. 2008. Landscape evolution and climate changes in the Late Pleistocene-Holocene, southern Pampa (Argentina): Evidence from palynology, mammals and sedimentology. Quarter Int 181: 123-138.

Rabassa, J., Coronato, A.M., Salemme, M. 2005. Chronology of the late Cenozoic Patagonian glaciations and their correlation with biostratigraphic units of the Pampean region (Argentina). J S Am Earth Sci 20: 81-103.

Rambaut, A. 2009. FigTree v1. 3.1: Tree figure drawing tool. Website: http://tree. bio. ed. ac. uk/software/figtree.

Rambaut, A., Suchard, M., Xie, D., Drummond, A. 2014. Tracer v1. 6. Available at: http://beast. bio. ed. ac. uk/Tracer.

Reguero, M.A., Candela, A.M., Alonso, R.N. 2007. Biochronology and biostratigraphy of the Uquia Formation (Pliocene–Early Pleistocene, NW Argentina) and its significance in the Great American Biotic Interchange. J S Am Earth Sci 23: 1-16.

Reig, O., Busch, C., Ortells, M., Contreras, J. 1990. An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. In: Evolution of subterranean mammals at the organismal and molecular levels (Nevo, E., Reig, O.A. eds.) Prog Clin Biol Res 335: 71-96. Wiley-Liss, New York.

Rivera, D.S., Vianna, J.A., Ebensperger, L.A., Eduardo Palma, R. 2016. Phylogeography and demographic history of the Andean degu, Octodontomys gliroides (Rodentia: Octodontidae). Zool J Linn 178: 410-430.

Roratto, P.A., Fernandes, F.A., Freitas, T.R. 2015. Phylogeography of the subterranean rodent Ctenomys torquatus: an evaluation of the riverine barrier hypothesis. Journal of Biogeography, 42: 694-705.

Rosenberg, N.A. 2002. The probability of topological concordance of gene trees and species trees. Theor Popul Biol 61: 225-247.

Rosi, M.I., Scolaro, J.A., Videla, F. 1992. Distribución y relaciones sistemáticas entre poblaciones del género *Ctenomys* (Rodentia: Ctenomyidae) de la Provincia de Mendoza (Argentina). Miscellánia Zoologica 16: 207-222.

Rosi, M.I., Cona, M.I., Roig, V.G. 2002. Estado actual del conocimiento del roedor fosorial *Ctenomys mendocinus* Philippi 1869 (Rodentia: Ctenomyidae). Mastozoologa Neotropical 9: 277-295.

Rosi, M.I., Cona, M.I., Roig, V.G., Massarini, A.I., Verzi, D.H. (2005). *Ctenomys mendocinus*. Mammalian Species 1-6.

Shaffer, H.B., Thomson, R.C. 2007. Delimiting species in recent radiations. Syst Biol 56: 896-906.

Sistrom, M., Donnellan, S.C., Hutchinson, M.N. 2013. Delimiting species in recent radiations with low levels of morphological divergence: a case study in Australian *Gehyra* geckos. Mol Phylogenet Evol 68: 135-143.

Sites, J.W., Crandall, K.A. 1997. Testing species boundaries in biodiversity studies. Conserv Biol 11: 1289-1297.

Slamovits, C.H., Cook, J.A., Lessa, E.P., Rossi, M.S. 2001. Recurrent amplifications and deletions of satellite DNA accompanied chromosomal diversification in South American Tuco-tucos (Genus *Ctenomys*, Rodentia: Octodontidae): A phylogenetic approach. Mol Biol Evol 18: 1708-1719.

Stellatelli, O.A., Block, C., Vega, L.E., Cruz, F.B. 2014. Responses of two sympatric sand lizards to exotic forestations in the coastal dunes of Argentina: Some implications for conservation. Wildlife Res 41: 480-489.

Swofford, D.L. 2000. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer, Sunderland, Mass.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6:

molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725-2729.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876‑4882.

Tomasco, I.H., Lessa, E.P. 2011. The evolution of mitochondrial genomes in subterranean caviomorph rodents: Adaptation against a background of purifying selection. Mol Phylogenet Evol 61: 64-70.

Tomasco, I., Lessa, E. 2007. Phylogeography of the tuco-tuco *Ctenomys pearsoni*: mtDNA variation and its implication for chromosomal differentiation. In Kelt, D., Lessa, E., Salazar-Bravo, J., Patton, J., eds. The quintessential naturalist: honoring the life and legacy of Oliver P. Pearson, volume 134, pages 859-882. University of California Publications in Zoology Series, Berkeley, California.

Tonni, E.P., Cione, A.L., Figini, A.J. 1999. Predominance of arid climates indicated by mammals in the pampas of Argentina during the Late Pleistocene and Holocene. Palaeogeogr Palaeoc 147: 257-281.

Verzi, D., Olivares, A., Morgan, C. 2010. The oldest South American tuco-tuco (late Pliocene, northwestern Argentina) and the boundaries of the genus *Ctenomys* (Rodentia, Ctenomyidae). Z Saugetierkd 75: 243-252.

Vitullo, A., Roldan, E., Merani, M.S. 1988. On the morphology of spermatozoa of tuco-tucos, *Ctenomys* (Rodentia: Ctenomyidae): New data and its implications for the evolution of the genus. J Zool 215:675-683.

Zenuto, R., Busch, C. 1995. Influence of the subterranean rodent *Ctenomys australis* (tuco-tuco) in a sand-dune grassland. Z Saugetierkd 60: 277-285.

Figure legends

Figure 1: Sampling localities of *Ctenomys* in mid-central Argentina analyzed in this study. Shaded areas represent distributional ranges of species of tuco-tucos of the *C. mendocinus* species complex. The arrows pointed out the type localities for each taxon. Names and boundaries of Provinces in the Central Argentina are also shown.

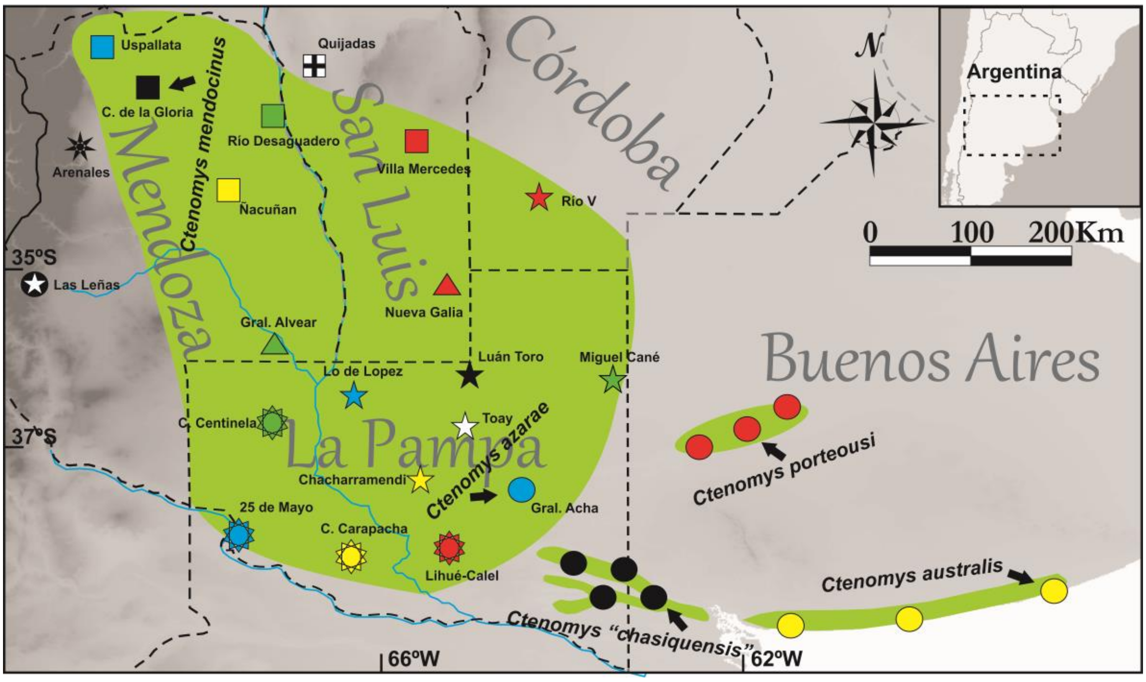
Figure 2: Phylogenetic tree resulting from maximum likelihood analysis from *Ctenomys* of mid-central Argentina based on mitochondrial sequences. Numbers indicate support from Maximum Likelihood, Bayesian and Neighbor Joining analyses. A node without numbers implies that support was lower than 50%. Shaded areas represent the three main clades identified.

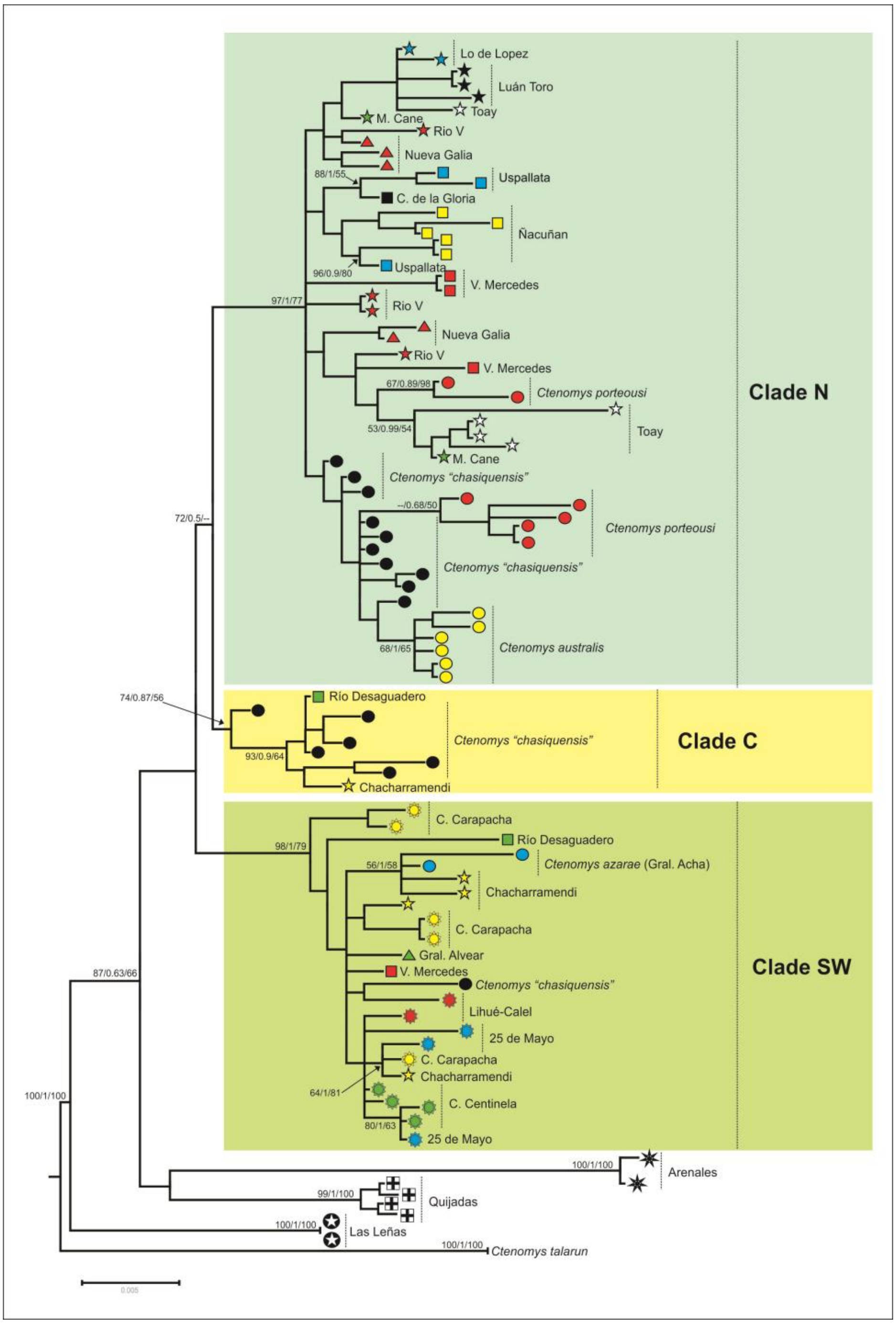
Figure 3: Median-joining haplotype network of concatenated sequences of mtDNA from populations of *Ctenomys* in mid-central Argentina. Symbol sizes are proportional to haplotype frequencies and the crossed marks are nucleotide substitutions inferred in that branch. Shaded areas represent the three main clades identified.

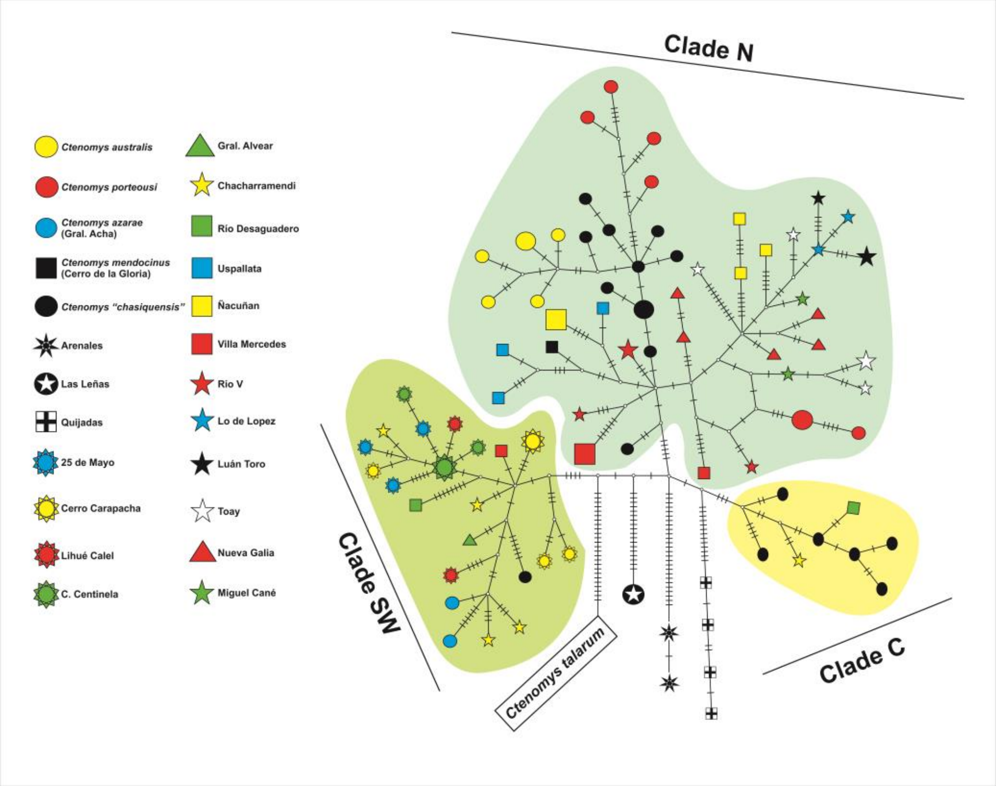
Figure 4: Relative frequencies of three major clades across sampling localities of *Ctenomys* in mid central Argentina.

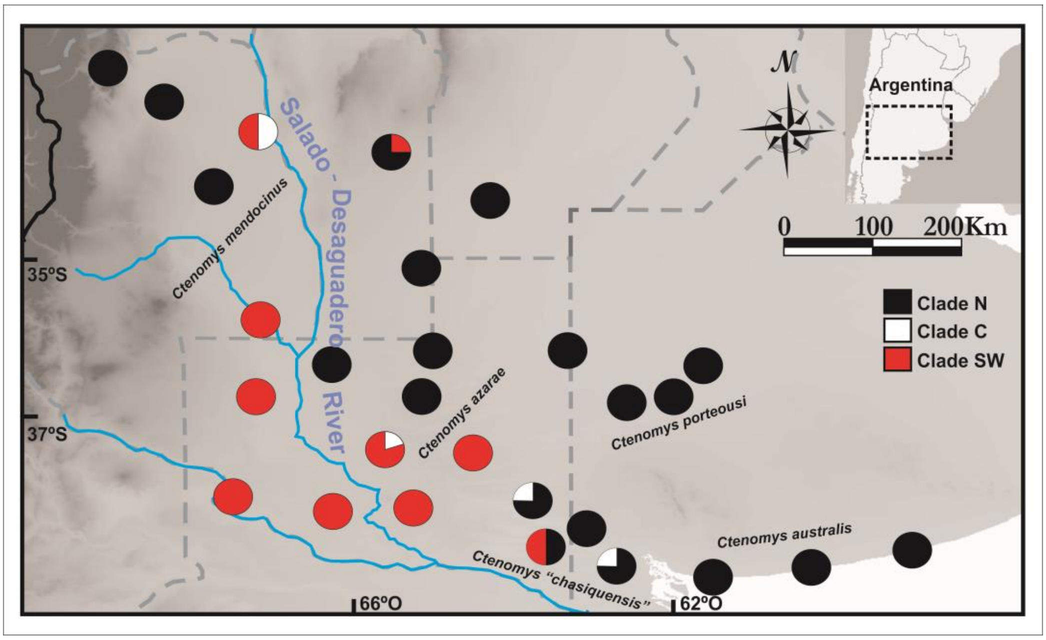
Figure 5: Historical demography inferred from *C. mendocinus* species complex. A: Mismatch pairwise distribution of mitochondrial sequences. Bars: observed distribution; dashed line: expected distribution under an exponential population expansion model. B: Bayesian skyline plot showing the effective population size fluctuations throughout time in *C. mendocinus* species complex. Black lines represent median estimations and the grey area represents the upper and lower 95% credible intervals. The x-axes was converted to time using a mutation rate of 4.42% Myr $^{-1}$ estimated for the mitochondrial control region of the genus *Ctenomys*.

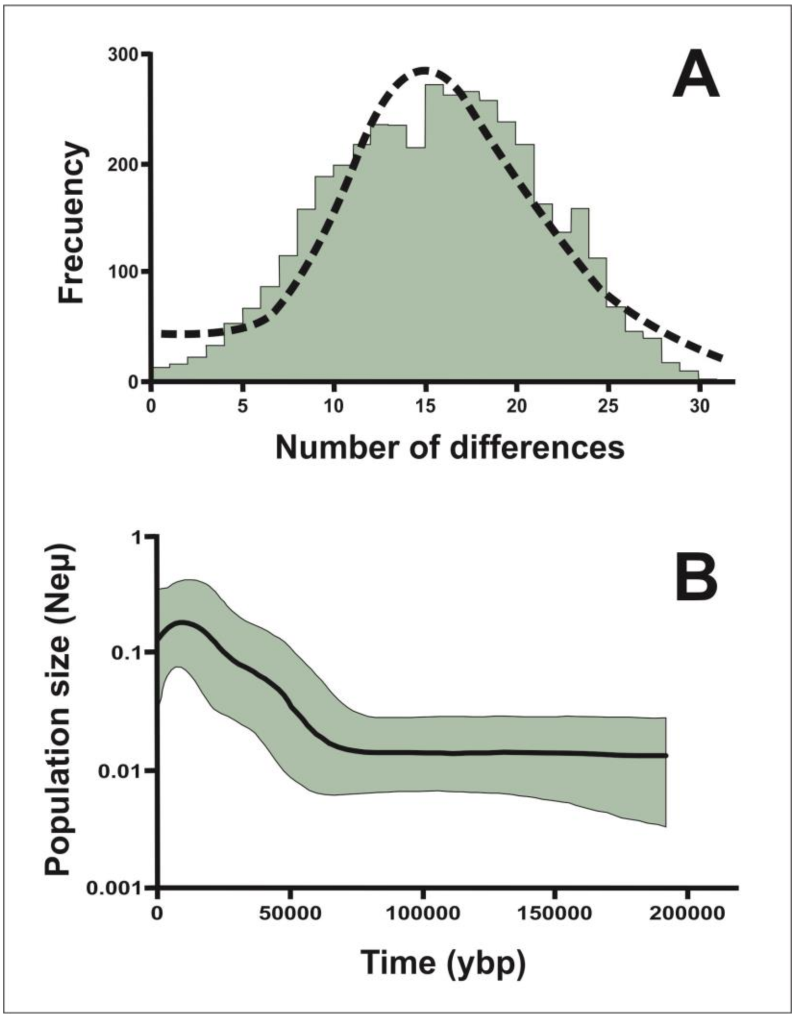
Figure 6: Genetic heat maps generated via the AIS software. Genetic topography varies from low (light colors) to high (dark colors) genetic distance values. Black points represent sampling localities.

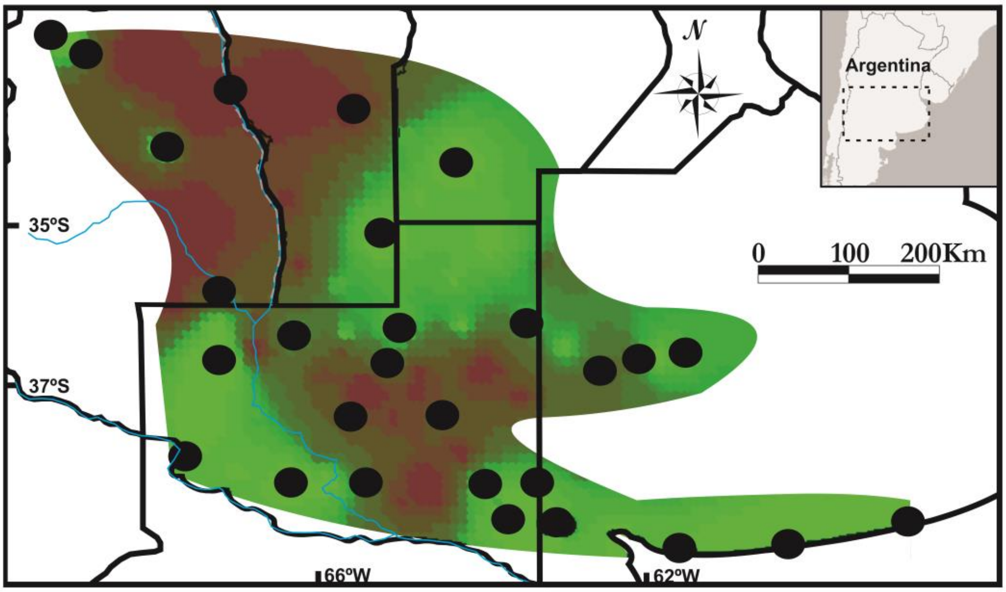

Uspallata
Quijadas
Córdoba
N
Argentina
C. de la Gloria
Ctenomys mendocinus
Río Desaguadero
San Luis
Arenales
Mendoza
Villa Mercedes
Ñacuñan
Río V
0
100
200Km
35ºS
Las Leñas
Nueva Galia
Gral. Alvear
Luán Toro
Miguel Cané
Buenos Aires
Lo de Lopez
Toay
C. Centinela
37ºS
La Pampa
Ctenomys azarae
Chacharramendi
Ctenomys porteousi
25 de Mayo
Gral. Acha
C. Carapacha
Lihué-Calel
Ctenomys australis
Ctenomys "chasiquensis"
66ºW
62ºW


Lo de Lopez
Luán Toro
Toay
M. Cane
Rio V
Nueva Galia
88/1/55
Uspallata
C. de la Gloria
Ñacuñan
96/0.9/80
Uspallata
V. Mercedes
97/1/77
Rio V
Nueva Galia
Rio V
V. Mercedes
67/0.89/98
Ctenomys porteousi
53/0.99/54
Toay
M. Cane
Ctenomys "chasiquensis"
--/0.68/50
Ctenomys porteousi
72/0.5/--
Ctenomys "chasiquensis"
68/1/65
Ctenomys australis
Clade N
74/0.87/56
Río Desaguadero
Ctenomys "chasiquensis"
93/0.9/64
Chacharramendi
Clade C
C. Carapacha
98/1/79
Río Desaguadero
56/1/58
Ctenomys azarae (Gral. Acha)
Chacharramendi
C. Carapacha
Gral. Alvear
87/0.63/66
V. Mercedes
Ctenomys "chasiquensis"
Lihué-Calel
25 de Mayo
C. Carapacha
Chacharramendi
64/1/81
C. Centinela
80/1/63
25 de Mayo
Clade SW
100/1/100
100/1/100
Arenales
99/1/100
Quijadas
100/1/100
Las Leñas
100/1/100
Ctenomys talarun
0.005


Ctenomys australis
Ctenomys porteousi
Ctenomys azarae (Gral. Acha)
Ctenomys mendocinus (Cerro de la Gloria)
Ctenomys "chasiquensis"
Arenales
Las Leñas
Quijadas
25 de Mayo
Cerro Carapacha
Lihué Calel
C. Centinela
Gral. Alvear
Chacharramendi
Río Desaguadero
Uspallata
Ñacuñan
Villa Mercedes
Río V
Lo de Lopez
Luán Toro
Toay
Nueva Galia
Miguel Cané
Clade N
Clade SW
Clade C
Ctenomys talarum


N
Argentina
0
100
200Km
Clade N
Clade C
Clade SW
Salado - Desaguadero River
Ctenomys mendocinus
Ctenomys azarae
Ctenomys porteousi
Ctenomys australis
Ctenomys "chasiquensis"
35ºS
37ºS
66ºO
62ºO


A
Frecuency
300
200
100
0
0
5
10
15
20
25
30
Number of differences
B
Population size (Neμ)
1
0.1
0.01
0.001
0
50000
100000
150000
200000
Time (ybp)



N
Argentina
0
100
200Km
35ºS
37ºS
66ºW
62ºW

TABLE 1: Divergence among sequence groups of *Ctenomys* for uncorrected p distance

C. talarum				
0.028	*C. mendocinus* species complex			
0.034	0.025	Arenales		
0.025	0.02	0.029	Las Leñas	
0.028	0.018	0.024	0.019	Quijadas

Clade N		
0.015	Clade SW	
0.012	0.012	Clade C

Appendix 1: Locality names, geographic location, sample size and Genbank accession numbers for the specimens of *Ctenomys* sampled throughout Central Argentina used in this study. Letters in parentheses indicate type locality for taxa analyzed. (a): *C. mendocinus*, (b): *C. azarae*, (c): *C. australis*, (d): *C. porteousi*, (e): *C. "chasiquensis"*.

Locality	Geographic location (Latitude - Longitude)	Sample size	Genbank accession number
Arenales	33.621ºS - 69.512ºW	2	xxxxx(pending)
Las Leñas	35.119ºS - 69.979ºW	2	xxxxx(pending)
Quijadas	32.807ºS - 66.786ºW	4	xxxxx(pending)
Uspallata	32.568ºS - 69.343ºW	3	xxxxx(pending)
Cerro de la Gloria (a)	32.914ºS - 68.904ºW	1	xxxxx(pending)
Río Desaguadero	33.325ºS - 67.174ºW	2	xxxxx(pending)
Ñacuñan	34.049ºS - 67.949ºW	5	xxxxx(pending)
Villa Mercedes	33.585ºS - 65.642ºW	4	xxxxx(pending)
Río V	34.216ºS - 64.385ºW	4	xxxxx(pending)
Nueva Galia	35.114ºS - 65.293ºW	5	xxxxx(pending)
General Alvear	35.813ºS-67.263ºW	1	xxxxx(pending)
Cerro Centinela	36.641ºS - 67.305ºW	4	xxxxx(pending)
Lo de Lopez	36.336ºS - 66.359ºW	2	xxxxx(pending)
Luán Toro	36.253ºS - 65.099ºW	3	xxxxx(pending)

Miguel Cané	36.176ºS - 63.528ºW	2	xxxxx(pending)
Toay	36.713ºS - 65.246ºW	5	xxxxx(pending)
25 de Mayo	37.844ºS - 67.714ºW	3	xxxxx(pending)
Chacharramendi	37.322ºS - 65.696ºW	5	xxxxx(pending)
Cerro Carapacha	38.143ºS - 66.401ºW	5	xxxxx(pending)
Lihué Calel	38.131ºS - 65.512ºW	2	xxxxx(pending)
General Acha (b)	37.352ºS - 64.569ºW	2	xxxxx(pending)
Ctenomys australis (c)	38.623ºS - 58.843ºW	2	xxxxx(pending)
	38.895ºS - 60.320ºW	2	xxxxx(pending)
	39.002ºS - 61.608ºW	2	xxxxx(pending)
Ctenomys porteousi (d)	36.793ºS - 62.308ºW	3	xxxxx(pending)
	36.810ºS - 62.605ºW	2	xxxxx(pending)
	36.576ºS - 61.658ºW	2	xxxxx(pending)
Ctenomys "chasiquensis" (e)	38.658ºS - 63.091ºW	5	xxxxx(pending)
	38.640ºS - 63.291ºW	4	xxxxx(pending)
	38.310ºS - 63.610ºW	4	xxxxx(pending)
	38.143ºS - 64.077ºW	5	xxxxx(pending)

Appendix 2: Number of sequences, polymorphic sites, number of haplotypes and haplotypic and nucleotidic diversity for the three main clades of *C. mendocinus* complex species.

Clade	Number of sequences	Number of polymorphic sites	Number of haplotypes	Haplotypic diversity	Nucleotidic diversity
N	59	76	49	0.993	0.006
C	8	16	8	1	0.004
SW	24	54	22	0.993	0.006