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Historical demography and spatial genetic structure of the subterranean rodent *Ctenomys magellanicus* in Tierra del Fuego (Argentina)

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Ctenomys (tuco-tuco) is the most numerous genus of South American subterranean rodents and one of the most genetically diverse clades of mammals known. In particular, the genus constitutes a very interesting model for evolutionary studies of genetic divergence and conservation. *Ctenomys magellanicus* is the southernmost species of the group and the only one living in Isla Grande de Tierra del Fuego (Argentina). This species presents two chromosomal forms (*Cm*34 and *Cm*36) fragmented into *demes* distributed from the north region (steppe) to the south region (ecotone) of the island, respectively; no hybrids or overlapping areas were detected. To study the historical demography and the spatial genetic structure of the *C. magellanicus* population we used mitochondrial DNA (mtDNA) (D-loop and cytochrome *b*) and microsatellite loci. Nine mtDNA haplotypes were identified, three of them belonging to the north and the other six to the south. Shared haplotypes between regions were not detected. mtDNA and microsatellite genotypes showed a marked pattern of population structure with low values of genetic flow between regions. The south is made up of small populations or isolated *demes* making up an endogamic metapopulation with unique alleles and haplotypes. Also, the results suggest a northward expansion process starting from an ancestral haplotype from the south. That population might have lived at a refuge through the adverse Pleistocene environmental conditions that took place at Tierra del Fuego. Results of this study are relevant to the conservation of *C. magellanicus*, suggesting that each region (north and south) might be considered as an Evolutionarily Significant Unit.

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ADDITIONAL KEYWORDS: conservation – demography – genetic – molecular markers – population.

INTRODUCTION

Subterranean rodents of the genus *Ctenomys* represent interesting models in evolutionary studies of genetic divergence (Reig *et al*., 1990; Giménez *et al*., 2002) and conservation biology (Shaffer *et al*., 2000; Cegelski, Waits & Anderson, 2003). Most species in this group present small effective population sizes and a marked spatial structure (Busch *et al*., 2000; Lacey, 2000; Fernández-Stolz, 2007; Fernández-Stolz, Stolz & Freitas, 2007; Mora *et al*., 2007, 2010). Moreover, some restrictions in habitat use generate discontinuous distributions (Mora *et al*., 2006, 2007) and fragmentation in most species, so it would also be possible that population structure patterns are determined mainly by local differentiation and limited genetic flow (El Jundi & Freitas, 2004; Opazo *et al*., 2008).

Ctenomys magellanicus is the southernmost species of the group, and the only subterranean rodent at Tierra del Fuego. A few years ago, it was considered

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a Vulnerable species by the IUCN, and accordingly was included in the Red Book of Argentine Mammals of SAREM (Lizarralde, 2000; Bidau, Lessa & Ojeda, 2008). With regards to its chromosomes, it is a polytypic species with two forms, *Cm*34 and *Cm*36, which differ in structural rearrangements. Hypothetically, it represents a case of active chromosomal speciation (Lizarralde *et al*., 2001, Lizarralde, Bolzán & Bianchi, 2003; Fasanella, 2012a). The population is fragmented into *demes* distributed from the north to the south of the island (Patagonia and Sub-Antarctic biogeographical provinces, respectively, after Cabrera & Willink, 1973). Even when extensive surveys have been performed, no hybrids or overlapping areas have been found, suggesting an absence of genetic flow between the two forms (Lizarralde *et al*., 2001, 2003).

Ctenomys magellanicus is currently distributed from the northern shore of Fagnano lake to northern San Sebastián bay (Fasanella, 2012a). However, its historical distribution might have also comprised southern Patagonia, in addition to Isla Grande de Tierra del Fuego, before the latter was separated from the continent. The oldest known *C. magellanicus* fossil records in southern Patagonia come from the site Cueva de Milodon in Magallanes Province (Chile), with a radiocarbon dating of 13 500 ± 470 and 10 400 ± 300 ^{14}C years BP (Simonetti & Rau, 1989). However, the separation of the archipelago from the rest of the continent and the formation of the present Magellan Strait would have taken place some 10 200 cal years BP, when sea level ascended above 35 m (Ponce *et al*., 2011). Undoubtedly, the palaeoenvironmental setting of the area experienced fluctuating climatic conditions, with important consequences for the distribution, expansion, and availability of different environments and plant and animal resources (Rabassa *et al*., 2000; Borrero, 2001). During the Great Patagonian Glaciation, discharge glaciers were radially displaced and covered large sections of the Archipelago (Coronato, Meglioli & Rabassa, 2004) which might have limited the dispersal of *C. magellanicus* and restricted its distribution pattern towards the southern reaches of the island, where a steppe environment predominated.

Due to the difficulties associated with a direct quantification of migration in the field, little is known about the genetic structure and dispersal of this species. However, the use of indirect approximations such as genetic hypervariable markers [mitochondrial DNA (mtDNA) and microsatellite loci] brings up new opportunities to obtain reliable quantifications of dispersal rates and other aspects, which contribute in large part to a better understanding of evolutionary features for the species. Therefore, in this study were used the Control Region (D-loop), cytochrome *b* gene (Cyt *b*) and microsatellite loci with the aim of characterizing the population of *C. magellanicus* in Tierra del Fuego and explore the following aspects. (1) Does *C. magellanicus* belong to a monophyletic group? (2) What is their evolutionary status within the genus? (3) What is their genetic–spatial differentiation pattern from a historical demography perspective?

MATERIAL AND METHODS

Study area and sampling

Sixty tissue samples (blood, muscle, and liver) were analysed. The samples were deposited at the Tissue Samples Bank at Centro Regional de Estudios Genómicos of Universidad Nacional de La Plata, and came from previous studies from our group (Lizarralde *et al*., 2001, 2003; Fasanella, 2012a). Samples were selected from a pool so as to include six representative populations in the analysis, comprising the whole distribution range of this species in Tierra del Fuego. Specimen and sample collection followed current national and international research protocols and animal welfare standards.

In particular for the analysis performed in this study, population samples were assigned to two regions within the island, namely (1) north and (2) south, separated by the Rio Grande river. Previous studies (Reig & Kiblisky, 1969; Gallardo, 1991; Lizarralde *et al*., 2001, 2003) have indicated that the chromosome form *Cm*34 is restricted to the north, and form *Cm*36 to the south. Within each region were distinguished subpopulation groups identified for the molecular analysis as A and B in the north, and C–F in the south (Fig. 1).

PCR amplification of mitochondrial DNA

Total DNA was extracted with salts following the protocol of Aljanabi & Martínez (1997). Amplified sequences were the complete Cyt *b* gene (1140 bp) and a partial sequence of the D-loop (approx. 500 bp).

The Cyt *b* gene was amplified using a combination of primers MVZ 05 – L14115 (5′-CGAAGCTTGATA TGAAAAACCATCGTTG-3′) and MVZ14 – H15825 (5′-GGTCTTCATCTYHGGYTTACAAGAC-3′) (Smith & Patton, 1993). The D-loop was amplified using the TucoPro primers (5′-TTCTAATTAAACTATTTCTTG-3′, Tomasco & Lessa, 2006) and DL-H16340 (5′-CCTGAAGTAGGAACCAGATG-3′, Vilà *et al*., 1999). Amplification of the double-stranded product was performed in a total reaction volume of 25 μL with two PCR thermal profiles using *Thermus aquaticus* DNA polymerase. Twenty-five microlitres of PCR mix contained 1.25 U of *Taq* DNA polymerase (Invitrogen), 2.5 μL of 10× Tag polymerase buffer with $(NH_4)SO_4$, 1.5 mM of $MgCl_2$, 200 μM of DNTPs and 5 pmol of each primer. Thermal profiles of Cyt *b* consisted of 40

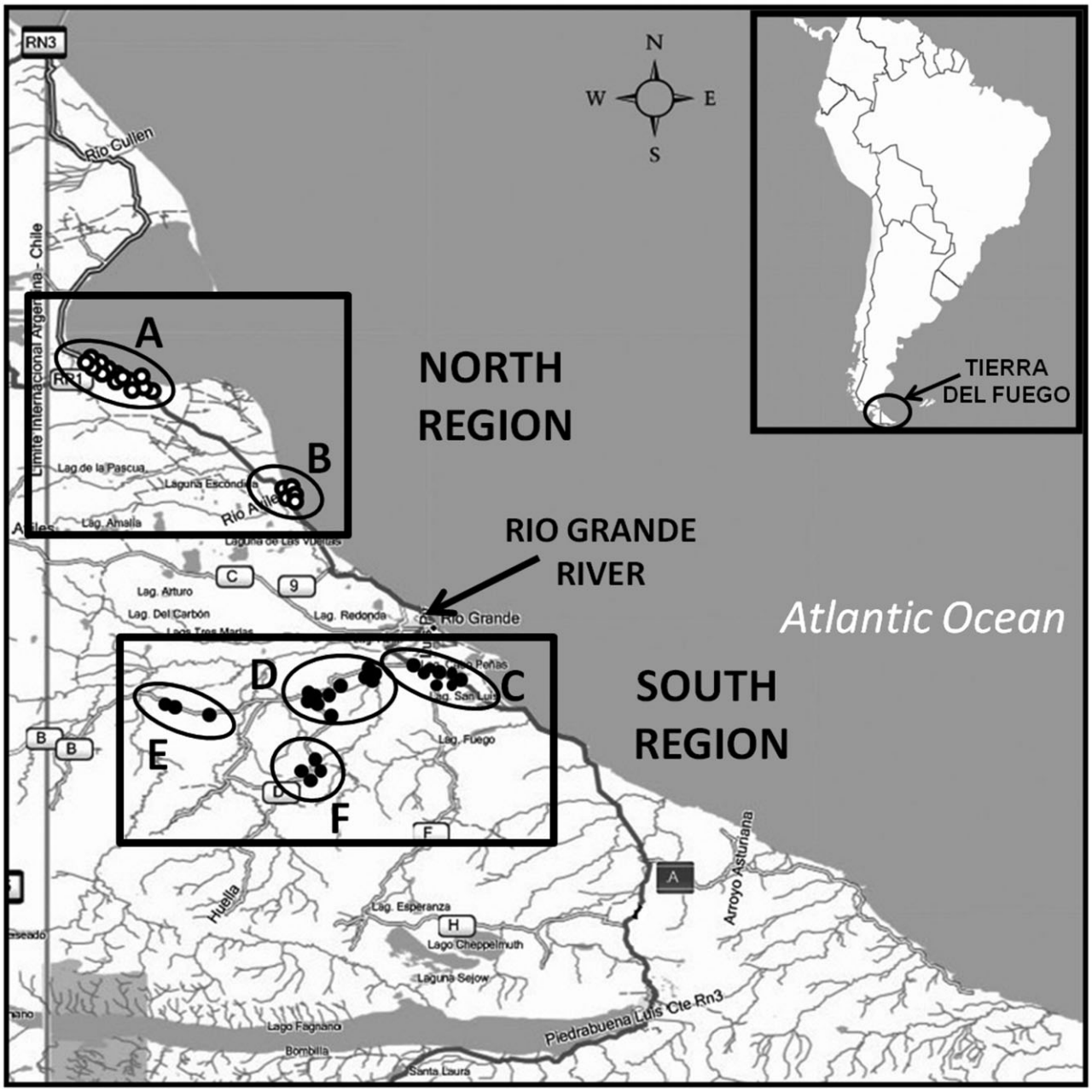

Figure 1. Geographical distribution of *Ctenomys magellanicus* sampling sites along the study area. Squares show the two regions: north (steppe, chromosome form $2n = 34$) and south (ecotone, chromosome form $2n = 36$). Each region was subdivided into subpopulations: two for the north (subpopulations A and B) and four for the south (subpopulations C–F).

cycles, each with 1 min denaturation at 94 °C, 1.5 min annealing at 55 °C, and 2–6 min extension at 72 °C. Thermal profile for the D-loop consisted of an initial denaturation step at 94 °C for 4 min followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 74 °C for 40 s, and finally a final extension at 74 °C for 6 min. Both chains (forward and reverse) were automatically sequenced in an ABI3100 sequencer (Macrogen, Inc., Korea). Chromatograms were edited using the program BIOEDIT 7.0 (Hall, 1999). The sequences were aligned using Clustal X (Thompson *et al*., 1997).

PCR amplification and screening of microsatellites

We amplified five microsatellite loci originally designed for *Ctenomys haigi* (Hai8 and Hai11; Lacey & Maldonado, 1999) and *Ctenomys sociabilis* (Soc4, Soc5 and Soc6; Lacey, 2001) that proved to be polymorphic in *C. magellanicus*, in 20 individuals from each region (i.e. north and south). PCR amplifications were performed using fluorescently labelled primers and carried out in a reaction volume of 15 μL containing 50–150 ng of DNA, 1× Buffer *Taq* Polymerase, 1.5 mM Cl_2Mg, 200 μM of each dNTP, 25 mM of each primer and 1.25 U of *Taq* Polymerase (UNQUI PB-L). Thermal cycling for PCR consisted of initial denaturation at 94 °C for 5 min, followed by 34 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56–64 °C (depending on the primer pair used), and 45 s extension at 74 °C, and a final extension at 74 °C for 5 min. The final PCR fluorescently labelled products were analysed with a capillary sequencer ABI3100 (Macrogen). Considering the relative sizes of fragments and the number of dye labels, we used the following combination of multiplexing for genotyping: Hai8/Hai11/Soc4 and Soc5/Soc6. Readings were performed with the software PeakScanner 1.0 (Applied Biosystems).

Phylogenetic analyses

A molecular phylogenetic approach was used to investigate the evolutionary position of specimens identified as *C. magellanicus* from Tierra del Fuego. In the two molecular analyses, we used all available sequences of *Ctenomys* from GenBank. Datasets used are not the same for Cyt *b* and D-loop. Sequences of *Tympanoctomys barrerae*, *Octodon degus*, *Spalacopus cyanus*, *Aconaemys fuscus*, and three species of *Proechimys* were used as outgroup.

The final alignments comprised 279 sequences of *Ctenomys* for Cyt *b* and 257 for the D-loop. Sequences were aligned manually using BioEdit (Hall, 1999). First, separate analyses were conducted on the two molecular markers, appropriate substitution models were estimated with the Akaike information criterion (AIC) as implemented in MrAIC (Nylander, 2004). Two phylogenetic reconstruction methods were analysed.

1 Maximum-likelihood (ML) phylogenetic reconstructions were performed using TreeFinder (Jobb *et al.*, 2004). Confidence values for the edges of the ML tree were computed by bootstrapping (Felsenstein, 1985) with 1000 replications.

2 Bayesian inference (BI) analysis was conducted in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Four chains were run simultaneously (three heated, one cold) for 20 000 000 generations, with tree space sampled every 100th generation. After a graphical analysis of the evolution of the likelihood scores, the first 300 000 generations were discarded as burn-in. The remaining trees were used to calculate the consensus tree.

Genetic structure and historical demographic

mtDNA

Genetic diversity was estimated from haplotype (h) and nucleotide (π) diversity using the software DNAsp 4.10 (Rozas *et al.*, 2003). A minimum spanning tree (Excoffier & Smouse, 1994) was generated using Arlequin 3.11 (Excoffier, Laval & Schneider, 2005). Fu's (Fu, 1997) and Tajima's (Tajima, 1989) neutrality tests were carried out to analyse the historical demography of *C. magellanicus*. Such tests were run together with plots of pairwise differences, 'mismatch distributions' (Rogers & Harpending, 1992), and evidence of demographic changes and/or deviations from neutrality. Significantly negative values of Tajimas's *D* and Fu's *Fs* are indicative of an excess of recent mutations, relative to expectations under the standard neutral model. Such analyses were performed using the software DNAsp 4.0 (Rozas *et al.*, 2003). We examined the demographic history of populations by an analysis of 'mismatch distributions', which is able to distinguish between populations that have been stable over time from those that have experienced recent expansion or reduction (Rogers & Harpending, 1992; Su *et al.*, 2001).

The geographical differentiation of north and south populations was determined by the hierarchical analysis of molecular variance (AMOVA) with the software GenAIEx 6.0 (Peakall & Smouse, 2006). Finally, to evaluate a possible 'isolation by distance' pattern (Slatkin, 1993), a Mantel test was performed (Mantel, 1967).

Microsatellites

The genetic diversity within each population was measured as the number of alleles per locus (N_A), the average number of alleles (A), and expected (H_e) and observed (H_o) heterozygosity, using Arlequin 3.11 (Excoffier *et al.*, 2005) and GenAIEX 6.0 (Peakall & Smouse, 2006). Additionally, analysis of linkage disequilibrium between pairs of loci was carried out using GenAiEX 6.0 (Peakall & Smouse, 2006), and deviations from Hardy–Weinberg (H-W) equilibrium were tested with Arlequin 3.11 (Excoffier *et al.*, 2005). Fisher's exact test was performed through Markov chains (Raymond & Rousset, 1995) with 5000 iterations, applying Bonferroni's sequential correction. We used the software Cervus 3.0 (Kalinowski, Taper &

Marsahll, 2007) to evaluate the presence of null alleles within each sampling site, taking into account a cut-off point of 0.05. Regions north and south were simultaneously considered in the study of both null alleles and linkage disequilibrium.

The software STRUCTURE (Pritchard, Sthephens & Donelly, 2000) was used to estimate the probable number of populations (k) to which samples were later assigned; 50 000 initial burn-in steps were considered, followed by 100 000 simulations. Ten independent runs were performed for each possible k (k = 1, 2, 3, 4, 5, and 6), considering a cutout value of $Q > 0.7$ for the assignment of individual samples to populations. To determine correct k values, the software STRUCTURE HARVESTER was used (Earl, 2011). The spatial structuring of genetic groupings defined by STRUCTURE was analysed by means of AMOVA, using the software GenAIEx 6.0 (Peakall & Smouse, 2006).

RESULTS

Phylogenetic analysis

Cytochrome b

Sequence alignment of the Cyt *b* gene comprised 1140 bp positions. However, because not all sequences completed the 1140 bp, this alignment had 29% missing data. The model of sequence evolution that best fitted our sequence data set was HKY + gamma, according to MrAIC (Nylander, 2004). The ML and BI phylogenetic trees obtained had similar topologies. The BI tree with the support values of the two analyses is shown in Figure 2. The four sequences corresponding to *C. magellanicus* are grouped in a single

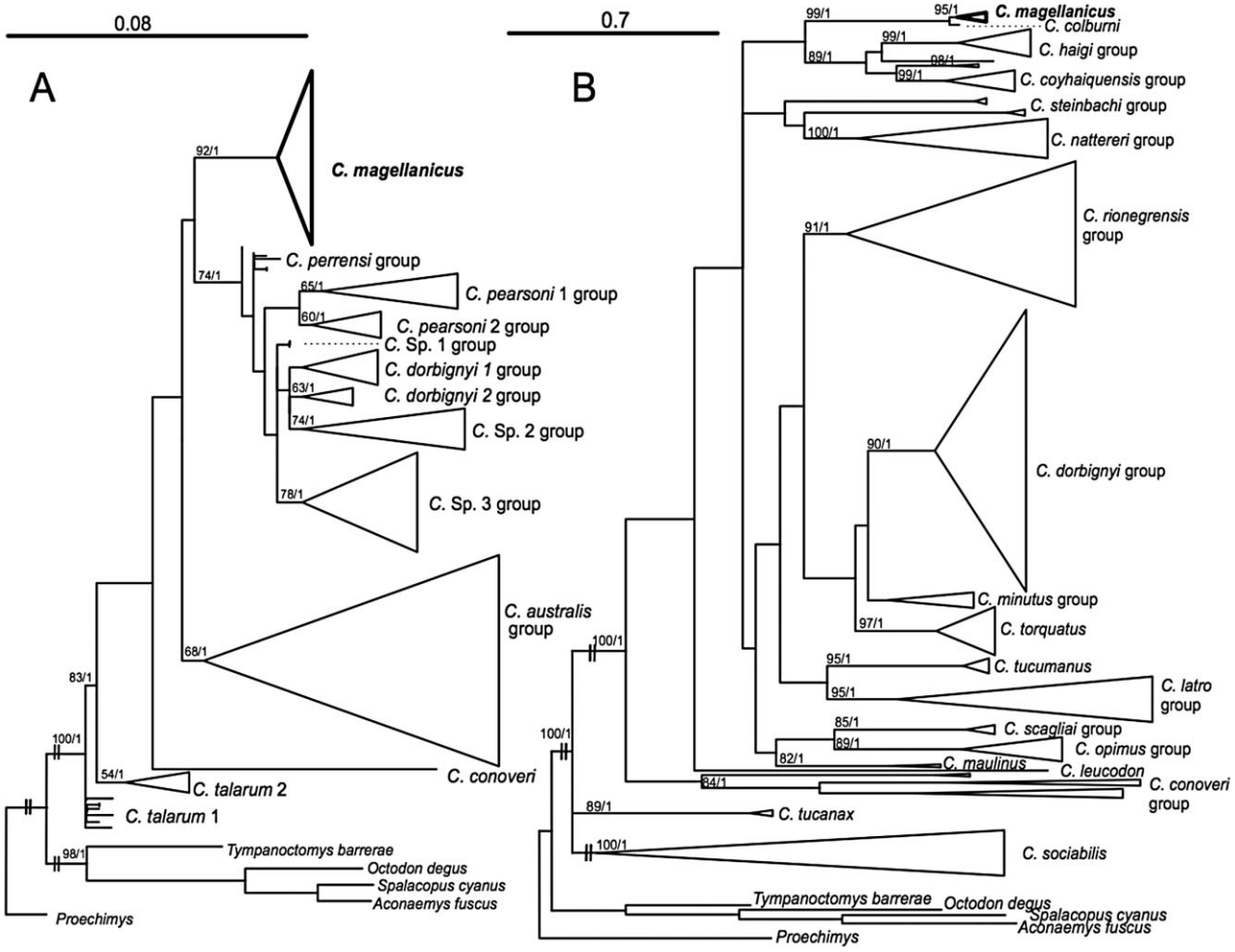

Figure 2. Bayesian inference trees of *Ctenomys* genus. A, tree derived from the D-loop marker. B, tree derived from Cyt *b*. Numbers next to branches are bootstrap support values and Bayesian posterior probabilities, respectively.

clade (95/1 bootstrap and posterior probabilities, respectively) forming a monophyletic group. *Ctenomys colburni* is associated with these sequences, therefore appearing as the sibling species to *C. magellanicus* (with 99/1 bootstrap and posterior probabilities, respectively). These two species belong to a well-sustained group (74 bootstrap) including representatives from the following species: *C. coyhaiquensis*, *C. fodax*, *C. sericeus*, *C. haigi*, and several unidentified *Ctenomys* species. The other species do not present good support values.

Control region

Sequence alignment of the D-loop comprised 454 bp. Not all used sequences completed the 454 bp, but this alignment had only 7% missing data. The model of sequence evolution that best fitted our sequence data set was HKY + gamma, according to MrAIC (Nylander, 2004). The ML and BI phylogenetic trees obtained had similar topologies (Fig. 2). All the haplotypes corresponding to *C. magellanicus* form a well-supported monophyletic clade (92/1 bootstrap and posterior probabilities, respectively). However, we cannot discern with certainty which one represents the sibling group to the species, given that no interspecific relationships or species groups are supported by adequate statistics.

Both phylogenetic analyses supported the monophyly of *C. magellanicus* in Tierra del Fuego.

Population genetic analyses based on mitochondrial sequences of the D-loop

Nine D-loop haplotypes were identified (GenBank accession numbers HQ262415–23) differing in 11 change sites, 10 transitions (T/C type) and one transversion (A/G type). No indels were found. Of these nine haplotypes, three corresponded exclusively to the north while the other six corresponded to the south (Table 1, Fig. 1). The most frequent haplotypes were H5 in the north and H6 in the south (Table 1, Fig. 3A); also, H6 could be the ancestral haplotype given its central position in the spanning tree. The topology of the minimum spanning tree connecting mtDNA haplotypes is 'star-like'; haplotypes with relatively high frequencies (H5, H6) are represented in many populations.

Haplotype diversity (h) was 0.836, while nucleotide diversity (π) was 0.004 for the total number of analysed samples. Haplotype diversity was greater in the south ($h = 0.715$) than in the north ($h = 0.653$). Nucleotide diversity values for the south and north were similar (0.004 and 0.003, respectively) and are related to the number of nucleotide differences between the haplotypes ($k = 1.959$).

Both Tajima's D and Fu's Fs tests were positive and not significant, considering either both regions jointly ($D = 0.838$, $P > 0.100$; $Fs = 0.950$, $P = 0.123$) or separately (north: $D = 1.05$, $P > 0.100$; $Fs = 1.997$, $P = 0.165$; south: $D = 0.577$, $P > 0.100$; $Fs = 0.545$, $P = 0.178$). However, mismatch distribution followed an unimodal pattern, suggesting a recent history of demographic expansion, also evidenced by the low average value of nucleotide differences ($k = 1.959$) that characterizes these demographic events (Fig. 3B). When the regions were analysed separately, the distribution was unimodal, with few nucleotide differences ($k = 1.756$ in the south; $k = 1.455$ in the north).

AMOVA showed that the difference between south and north was 18%, and 29% between populations. The larger percentage variance was distributed within populations (53%).

The parameter Φ_{st} (used for haploid markers) measured between north and south was 0.181 ($P = 0.001$), while among populations it was 0.352 ($P = 0.001$). All populations from the south region presented Φ_{st} values larger than 0.3 that were significant (with the exception of the population pair C–D, $P = 0.069$), indicating that, unlike the north, the south population is strongly structured, with a value of 0.035 ($P = 0.365$) for Φ_{st} (Table 2), revealing a low degree of genetic structuring.

Table 1. Haplotype number and the distribution of each haplotype by population (A–F) and region (south, north)

		Haplotypes									
Region	Population	1	2	3	4	5	6	7	8	9	Total
North	A	5				11		9			25
	B					4		1			5
	C		5	3			4				12
South	D						10		1		11
	E									3	3
	F				3		1				4
Total		5	5	3	3	15	15	10	1	3	60

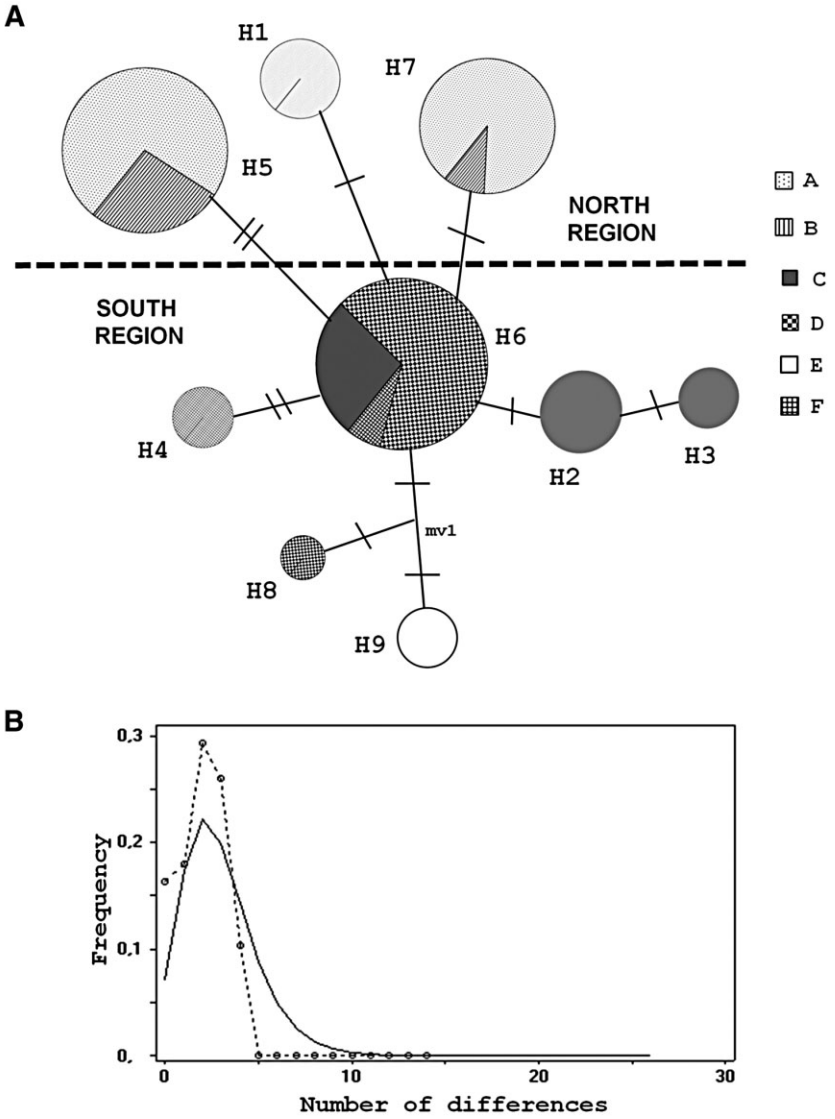

Figure 3. A, minimum spanning tree of nine mtDNA haplotypes of *Ctenomys magellanicus* from Tierra del Fuego, Argentina. Areas are proportional to haplotype frequencies, shading indicates populations, and cross hatches represent nucleotide differences between haplotypes. Haplotype numbers correspond to those of Table 1. Abbreviations for populations are given in Figure 1. B, observed and expected mismatch distributions for *C. magellanicus* (south + north). Dashed line, observed distribution; solid line, theoretical expected distribution under a population expansion model.

Mantel's test was not significant ($r = -0.103$, $P = 0.385$), indicating that the population does not fit into a model of 'isolation by distance' (Fig. 4).

Population genetic analyses based on microsatellites

Only one locus (Soc5) of the five studied presented a non-significant frequency of null alleles ($F = -0.023$, $P > 0.050$); the remaining loci presented significant frequencies of null alleles ($F_{Soc6} = 0.243$, $P < 0.005$; $F_{Soc4} = 0.192$; $P < 0.005$, $F_{Hai8} = 0.311$, $P < 0.005$; $F_{Hai11} = 0.081$, $P < 0.005$). The Soc5 locus was the only one that showed an excess of heterozygote genotypes. When the two regions were analysed separately, the Soc5 locus continually presented a higher frequency of heterozygotes ($F_{North} = -0.103$, $P > 0.005$; $F_{South} = 0.014$, $P > 0.005$). The remaining loci presented significant frequencies of null alleles only in the south ($F_{Soc6} = 0.195$, $P < 0.005$; $F_{Soc4} = 0.540$, $P < 0.005$; $F_{Hai8} = 0.479$, $P < 0.005$; $F_{Hai11} = 0.053$, $P < 0.050$), while in the north the frequencies were non-significant ($F_{Soc6} = 0.018$,

Table 2. Pairwise Φ_{st} estimates among the six populations (below the diagonal) and significance probability (above the diagonal)

		North		South			
Region	Subpopulation	A	B	C	D	E	F
North	A	–	0.365	0.001	0.001	0.002	0.001
	B	0.035	–	0.001	0.001	0.027	0.018
South	C	**0.316**	**0.403**	–	0.002	0.009	0.026
	D	**0.520**	**0.747**	**0.350**	–	0.002	0.006
	E	**0.481**	**0.742**	**0.476**	**0.854**	–	0.069
	F	**0.383**	**0.555**	**0.304**	**0.643**	0.707	–

Significant Φ_{st} values (< 0.05) are shown in bold.

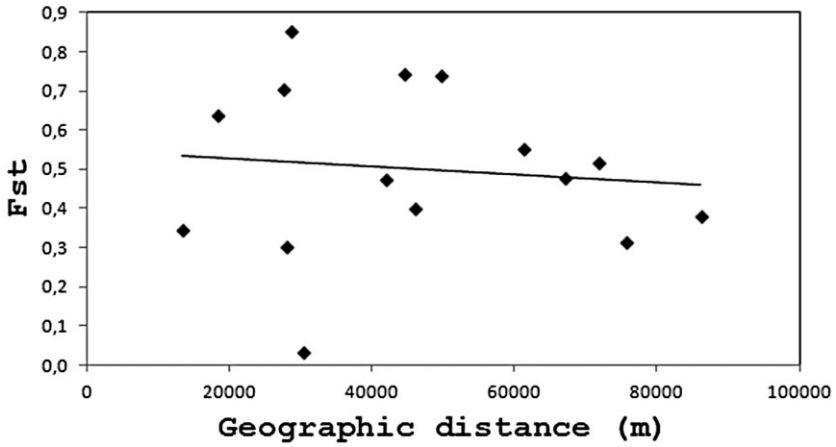

Figure 4. Relationship between pairwise geographical distances and F_{st} for *Ctenomys magellanicus* from Tierra del Fuego, based on F_{st} from mitochondrial control region sequences. The relationship between variables was non-significant (see Results).

$P > 0.050$; $F_{Soc4} = -0.011$, $P > 0.059$; $F_{Hai8} = 0.098$, $P > 0.050$; $F_{Hai11} = 0.044$, $P > 0.050$).

Only five pairwise comparisons revealed linkage disequilibrium, and they were not associated with any loci in particular (Soc5–Soc6 $P = 0.018$; Soc6–Hai8 $P = 0.001$; Soc4–Hai8 $P = 0.002$; Soc6–Hai11 $P = 0.001$; Hai8–Hai11 $P = 0.001$). Similarly to other studies with *Ctenomys*, these few microsatellites do not present significant deviations from linkage (Cutrera, Lacey & Busch, 2005, 2006; Mora, 2008; Mapelli, 2010). Therefore, the five loci used in this study were considered as 'independent markers'.

All loci were polymorphic, resulting in between five and 15 alleles per locus, and an effective number between 2.898 and 4.432 for north and south populations, respectively (Table 3). The average number of alleles per locus was 9.8; the north population presented a smaller number of alleles per locus (6), while the south population presented an average of 7.4 alleles per locus. It is noteworthy that H_e was always greater than H_o, except for locus Soc5 (in the north) which presented a larger quantity of observed heterozygotes than expected, and therefore no significant deviation from H-W equilibrium was detected. The remaining loci presented significant deviations ($P < 0.05$; Table 3).

Population structure

Within the study area, it was possible to identify population structuring, resulting in a grouping value of $k = 4$ (STRUCTURE). Using the model proposed by Evanno, Regnaut & Goudet (2005), the grouping value that best fitted the data was $k = 3$. Even when a grouping value of $k = 3$ does not represent the maximum posterior probability value [ln$P(D)$], this model is sustained by the maximum value of ΔK (Evanno *et al.*, 2005), which implies the presence of three genetically different groups. Moreover, 85% of the data were assigned with a value of $Q > 0.7$, while

Table 3. Microsatellite genetic variation in *Ctenomys magellanicus* for each region

		North region				South region			
Locus	At	Ni	Ne	Ho	He	Ni	Ne	Ho	He
SOC4	5	4	1.713	0.400	0.416	3	1.512	**0.100*****	0.339
SOC5	10	8	3.883	0.900	0.743	8	5.333	0.800	0.813
SOC6	9	5	1.536	**0.350***	0.349	8	4.020	**0.500****	0.751
HAI8	15	9	5.226	**0.667****	0.809	10	7.018	**0.300*****	0.858
HAI11	10	4	2.133	0.500	0.531	8	4.278	**0.700****	0.766
Mean				0.600	0.570			0.800	0.705
A	9.8	6	2.898			7.4	4.432		
%P		100				100			
Nt		20				20			

Number of alleles per locus (A_t), number of alleles per region (N_i), number of effective alleles per region (N_e), observed heterozygosity (H_o) and Nei's estimated heterozygosity (H_e), per locus for each region (north and south). Mean, average over five loci; *A*, mean number of alleles total and per region (allelic richness); %P, percentage of polymorphic loci; N_t, total sample size. Significant deviations between observed and expected levels of heterozygosity in each region and locus by locus are shown. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$.

if $k = 4$ only 62.5% of the data would have been assigned with a value of $Q > 0.7$. Each genetic grouping had a different number of samples or individuals: the first grouping with $n = 18$ (GROUP 1), of which 89% ($n = 16$) belonged to south, presented a value of $Q \geq 0.75$. The second grouping, with $n = 5$ (GROUP 2), of which 100% belonged to the north, presented a value of $Q > 0.9$; this latter grouping was strongly consolidated. Finally, the third grouping presented $n = 17$ (GROUP 3) from both regions: 65% ($n = 11$) from the north with a value of $Q \geq 0.78$, and 12% ($n = 2$) from the south with a value of $Q \geq 0.7$. Six individuals could not be clearly assigned to any genetic grouping ($Q < 0.7$); in particular, two belonged to the first grouping and the other four to the third.

When analysing F_{is} and H-W equilibrium values, only the grouping spatially related to the south presented significant deviations from H-W equilibrium (loci Soc4, Soc6, Hai8, and Hai11) and positive and significant F_{is} values ($F_{is} = 0.314$, $P < 0.001$). The other two groupings related to the north presented small values of F_{is} that were not significant ($F_{is} = 0.090$, $P = 0.572$; and $F_{is} = 0.003$, $P = 0.189$). Therefore, this region might not present endogamy, and each population might behave panmictically. The AMOVA performed for the groupings defined by STRUCTURE (which does not consider the two migrant groups) indicated that the greater variance was within each population (70%). The difference among the three groupings or populations (GROUPS 1, 2 and 3) was 11%, while the difference between regions was 19%, and the F_{st} value between them was 0.108 ($P = 0.001$), and F_{is} value was 0.212 ($P = 0.001$). Finally, the mismatch distribution of the north population produced a low, non-significant F_{st} value ($F_{st} = 0.009$, $P = 0.281$), while between the north and south populations, the F_{st} value was > 0.1 and significant ($F_{stGROUP\ 2\ -\ GROUP\ 1} = 0.140$, $P = 0.001$; $F_{stGROUP\ 3\ -\ GROUP\ 1} = 0.116$, $P = 0.001$). These results are similar to those obtained with mtDNA, suggesting a greater effect of genetic drift and therefore a greater F_{st} value between pairs of populations coming from different regions, resulting in a lowest effect (and therefore more genetic flow) within populations belonging to the same region.

DISCUSSION

Phylogenetic analysis

The phylogenetic trees obtained in the genus *Ctenomys* allow us to analyse different aspects of its evolution. We mainly see that *C. magellanicus* forms a monophyletic group with unique molecular characteristics that reaffirm its specific status. This does not seem to be so in other groups, which are composed of individuals from different species and/or individuals of uncertain specific identity. The other species of *Ctenomys* do not present good support values so they cannot be defined as clades, and it is beyond the scope of this work to discuss them.

Furthermore, geographically the analysis of all available samples enabled us to confirm that, thus far, *C. magellanicus* only inhabits Tierra del Fuego because we found no shared haplotypes on the continent, and therefore it was not possible accurately to determine the evolutionary position of

C. magellanicus within the genus. The mitochondrial markers used probably do not allow us good resolution of the relationships among the different species. Note that our objective was not to determine taxonomic or geographical groups in *Ctenomys*, but rather to know more about those species groups related to *C. magellanicus*. The lack of resolution between different phylogenetic lineages in the genus could well be related to the radiation that has recently given rise to the great diversity of this specific rodent group.

Genetic variation

Study data report moderate values of genetic diversity for *C. magellanicus*, when compared with values obtained for other species in the genus *Ctenomys* (Fernández-Stolz, 2007; Mora, 2008). In particular, *C. magellanicus* presented a low nucleotide diversity ($\pi = 0.0043$) in the mitochondrial D-loop marker, and high haplotype diversity ($h = 0.836$), with closely related haplotypes. In fact, the average number of nucleotide changes was ~2 bp.

By contrast, variability levels found in microsatellite loci of *C. magellanicus* are among the highest for the genus *Ctenomys*. The average number of alleles per locus was 9.8, ranging between five and 15. Other species of the genus presented an average number of alleles per locus of 13 (Group *C. perrensi*, range 5–19; Mirol *et al*., 2010), 9.3 (*C. minutus*, range 5–15; Gava & Freitas, 2004), 7.5 (*C. haigi*, range 3–13; Lacey, 2001), and 4.3 (*C. australis*, range 3–6; Mora *et al*., 2010). This result could be used in conservation programmes (see Implications for the conservation of *C. magellanicus* in this section).

Historical demography and phylogenetic relationships

The genetic diversity pattern elucidated for *C. magellanicus* is consistent with a small effective population size, followed by population expansion (Grant & Bowen, 1998). Even if results from the neutrality tests were not significant, the plots from mismatch distribution indicate that the population of *C. magellanicus* went through an expansion period, as indicated by the unimodal distribution of observed frequencies. Moreover, the minimum spanning tree shows a 'star-like topology' consistent with a process of population expansion. The phylogenetic relationships expressed in the network indicate that expansion took place starting from an ancestral haplotype with a distribution restricted to the ecotone (south), and that it might have had a karyotype $2n = 36$. In that sense, the colonization of the area currently occupied by *C. magellanicus* might have followed a south to north direction. Interestingly, results suggest that the Río Grande river, which separates the two regions, did not act as a natural barrier for the species.

Undoubtedly, the historical demography revealed for *C. magellanicus* can be correlated with the environmental and climatic changes that took place mainly during the Quaternary, particularly those that have been preserved in the record for Tierra del Fuego. Such changes spanned the whole extension of the Isla Grande, from the eastern opening of the Magellan Strait, San Sebastián Bay, and the eastern mouth of the Beagle Channel (Coronato *et al*., 2004). In turn, Pleistocene refuges enabled the expansion of forests, which gradually occupied the areas that the receding glaciers vacated. The current limits of the forest–steppe ecotone became established after ~5000 BP (Markgraf, 1989). This palaeoenvironmental scenario probably favoured the south–north expansion of the population of *C. magellanicus* from Pleistocene refuges. Even if the Río Grande river currently separates the two regions, it does not seem to have acted as a geographical barrier in the distribution of this species during the Quaternary, when conditions were more arid than at present (Coronato, Borromei & Rabassa, 2007).

Genetic and geographical structure patterns

Results from this study point to a significant population structuring, with low values of genetic flow between regions, showing that the south comprises small populations or isolated demes making up an endogamic metapopulation with unique alleles and haplotypes. By contrast, the north presents a larger genetic flow, which might be related to the fact that individuals from one of its populations recently colonized the area (Fasanella, 2012a, b). Similar findings made by other authors also point that the absence of an 'isolation by distance' pattern in species with limited mobility, as is the case for underground rodents, is due to a recent expansion in the distribution area (Wlasiuk, Garza & Lessa, 2003; Mora *et al*., 2006).

The analysis of microsatellite loci also showed a high degree of agreement between the genetic groupings and the geographical location of the samples (with the exception of two samples from the south assigned to the north by Structure software), which might also point to a northward expansion process. Our data suggest that the south presents a higher level of endogamy and is isolated from the rest, which explains why the majority of loci in the south population had a significant frequency of null alleles.

Different mutation rates in the markers used in this study (those estimated for mtDNA range between 10^{-8} and 10^{-9} by generation site, Su *et al*., 2001; and for microsatellites they range between 10^{-3} and 10^{-4}, Matocq, 2004) allow for the interpretation of the data under the assumption that mitochondrial markers are usually good for inferring historical demography patterns, while microsatellite loci are useful in the estimation of recent demographic patterns (Dionne *et al*., 2008; Lada *et al*., 2008). Therefore, the results obtained in this study suggest that gene flow between the regions is greater today than in the past.

Our study confirms that *C. magellanicus* is presently fragmented into two populations in the Isla Grande of Tierra del Fuego. In particular, the population from the south ecotone is firmly structured and represents the ancestral population, given that it includes the basal haplotype of the species, suggesting that this population lived at a refuge through the adverse Pleistocene environmental conditions that took place at Tierra del Fuego. By contrast, the population from the steppe or north has low genetic structure and spans the most recently occupied area. Finally, the historical population expansion of *C. magellanicus*, together with other events such as the occupation and colonization of new habitats, allow us to infer that the population has not yet reached its demographic equilibrium.

Implications for the conservation of *C. magellanicus*

Genetic differentiation studies in fragmented landscapes are useful in the identification of appropriate management units and the determination of independent genetic units (Shaffer *et al*., 2000; Cegelski *et al*., 2003). Evolutionarily significant units (ESUs) are, according to Ryder (1986), population units that merit their own management and have a high conservation priority. By contrast, Moritz (1994) defines an ESU as a group of individuals or populations that present reciprocal monophyly for mitochondrial markers, and significant divergences in the allele frequencies of nuclear loci, attributable to populations, species, or subspecies, and also considering the kind of isolation of such populations. The concept of a management unit (MU; Moritz, 1994) was established for those cases in which reciprocal monophyly was not reached among lineages. This concept was originally defined for populations (or groups of populations) identified by a significant divergence in the allele frequencies of neutral loci (nuclear or mitochondrial), independently of the phylogenetic relationships between the alleles. Therefore, MUs are a group of individuals with a sufficiently low degree of ecological and genetic connectivity, which justifies a separate monitoring and management for each group (subpopulation; Palsbøll, Berubé & Allendorf, 2006).

The concepts discussed here might be applied to the conservation of *C. magellanicus*. In that sense, it has been suggested that each region (north and south) might be considered an ESU, given their high degree of isolation, the fact that no mitochondrial haplotypes are shared, and that they present different chromosomal number and nuclear differentiation. Therefore, and given that *C. magellanicus* presents two chromosomal forms, it should be necessary to conserve individuals in both regions. Appropriate MUs could be defined within each ESU for the conservation of these rodents in Tierra del Fuego.

Our results demonstrate that *C. magellanicus* presents a significant genetic and population structure, with limited genetic flow between the two regions and with differences within each, indicating that the south population is more genetically structured than the north population. Therefore, in the south, each subpopulation might be defined as an MU, given that they present high F_{st} values between them (i.e. great divergence between subpopulations; all divergence values between subpopulations were > 0.3). Moreover, the four subpopulations of the south presented unique haplotypes that were not shared either by subpopulations within the same region or with the north region (i.e. haplotypes 2 and 3 were only found in subpopulation C; haplotype 8 in subpopulation D; haplotype 9 in subpopulation E; and haplotype 4 in subpopulation F). Therefore, if any of these subpopulations disappeared, then these unique haplotypes would also disappear. On the other hand, given that subpopulation B is likely to have arisen from subpopulation A and that in the north the degree of divergence is not high (Fasanella, 2012a,b), we can considering population A as an MU for the north and thereby reduce conservation efforts.

Future management and conservation plans for this species should contemplate the genetic differentiation aspects presented in this study. If any of the five MUs identified by this study should become extinct, a great part of the genetic pool of the species would be lost.

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