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Original investigation

Genetic studies in the recently divergent *Eligmodontia puerulus* and *E. moreni* (Rodentia, Cricetidae, Sigmodontinae) from Puna and Monte deserts of South America

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ABSTRACT

Eligmodontia is a genus of phyllotine rodents adapted to arid environments with seven recognized species. The sister species *E. puerulus* and *E. moreni* are distributed in the adjacent highland Puna and lowland Monte deserts respectively, and show remarkable morphological and chromosomal differences. However, analyses of the cytochrome b gene showed important variability, without reciprocal monophyly between them. In order to study the evolutionary processes involved in the diversification of both taxa, we analyzed 1161 bp of the mitochondrial control region and flanking sequences (N = 60), as well as 759 bp of the first exon of the nuclear gene IRBP (N = 14). Individuals of both species from Jujuy, Catamarca and Mendoza Provinces of Argentina were previously karyotyped. Results showed that the mitochondrial sequences present high haplotype and nucleotide diversity within all population, and no haplotype was shared between both species. F_{ST} indicated that populations of both species were moderately structured. The network was constituted by two major haplogroups, one composed by *E. puerulus* samples from Jujuy, and the other composed of sequences of all studied populations. The Bayesian analysis showed three clusters, matching the network. Phylogenetic analysis recovered two clades with high support, in coincidence with the network groups. There was only one close join between sequences of both species, corresponding to samples from Catamarca. Thus, mitochondrial data suggested hybridization between both species in Catamarca, with asymmetric introgression. The IRBP showed low variability and, in the phylogenetic analysis, the sequences of *E. puerulus* form a monophyletic group with intermediate support, whereas those of *E. moreni* collapse into a basal polytomy. Our data indicated a recent divergence and absence of introgression in the nuclear genomes. The results at the population level with mitochondrial sequences, together with integrative taxonomy at the species level in a biogeographic context, suggest that climatic and geologic changes could have had an important role in the determination of genetic variability patterns observed in these rodents.

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Introduction

The most ancient and diverse subfamily of cricetid rodents in South America is Sigmodontinae. With approximately 380 described species, it is the largest group of rodents in this continent, which are found in virtually all habitats. This rich radiation was influenced by the complex historical geology of the region, which among others involved the elevation of the Andes, one of the highest mountain system in the world (Patton et al., 2015).

In the attempt to order the diversity of the Sigmodontinae in an evolutionary context, the subfamily was historically divided into several tribes. Recent phylogenetic studies support the existence of at least nine tribes, although there are several genera not included in the trival classification (Patton et al., 2015). Representatives of tribe Phyllotini are one of the most abundant and diverse rodents in South America deserts with direct Andean influence. In these regions, several species with important adaptations to arid environment were described (Díaz and Ojeda, 1999).

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Eligmodontia is a genus of small phyllotines distributed from high Andean altitude deserts of Bolivia and Peru, to the southern arid continental end in Argentina and Chile. The taxonomy of the genus was modified through the years and authors, as in most sigmodontines (Hershkovitz, 1962; Braun, 1993; Steppan, 1995; Steppan et al., 2007; Pariñas et al., 2014). Currently, seven species are recognized for the genus based on morphologic, chromosomal and molecular data (Patton et al., 2015). These species have a broad disjunct distribution, although several areas of sympatry were detected between some of them (Lanzone et al., 2007; Mares et al., 2008). *E. puerulus* and *E. moreni* are distributed in the high altitudes of the Puna desert, and the contiguous low altitudes of the Monte desert respectively, of Argentina and northern Chile; and despite its adjacent geographic ranges never were detected contact areas between them.

From the cytogenetic point of view, the chromosome complement of *E. puerulus* range from 2n = 31–37 (FN = 48) due to multiple Robertsonian rearrangements (Rb) that includes at least four fusions, and it is the most variable species at the chromosomal level described within phyllotines (Lanzone et al., 2016). In this species some populations have fixed chromosome numbers, whereas others are polymorphic (Lanzone et al., 2011). On the other hand, *E. moreni* has a constant and distinctive karyotype with 2n = 52 and FN = 50, which are the highest 2n and FN found in the genus.

At the morphological level, *E. puerulus* and *E. moreni* are differentiated at qualitative and quantitative univariate characters; and are also clearly distinct in the multivariate analysis (Lanzone et al., 2007, 2011). However, molecular studies based on the cytochrome b gene failed to recover both species as monophyletic, being polyphyletic in the gene trees obtained with this mitochondrial marker (Lanzone et al., 2011).

Incongruence between gene trees and species trees were observed in several taxa and can occur due to different causes such as: imperfect taxonomy, inadequate phylogenetic information, interspecific hybridization, and incomplete lineage sorting, among others. Most of these phenomena are more frequently observed in recently divergent lineages, and are directly associated to recent speciation events (Funk and Omland, 2003). Species split are one of the most studied topics in evolutionary biology, as it is considered the process that generated all planet's biodiversity.

Most investigations concerning speciation are focused in sister taxa of recent origin, because the primary characters and processes that initiated the divergence can be detected. In general, results showed that there is a high variability in *tempos* and modes of speciation in different taxa, indicating that several complex processes can lead species to split. Most biologists are in agreement with the idea that in most cases an isolation period is required to initiate the divergence. However, the degree of divergence required to maintain the species integrity after contact, the characters primarily involved in the divergence, and the degree of introgression in different characters subsequent to species hybridization, are still questions of intense investigations (King, 1993; Coyne and Orr, 2004; Martínez et al., 2010; Beysard and Heckel, 2014; Cyner et al., 2015).

In this sense, the main goal of this work is to study the genetic variability in populations of *E. puerulus* and *E. moreni* through the analyses of a highly variable mitochondrial marker (control region and flanking sequences). Also we analyzed a fragment of a nuclear marker (IRBP) of some selected individuals to compare mitochondrial and nuclear patterns of phylogenetic relationships among samples. Finally, the evidences were contrasted in order to determine whether both nominal species are independent evolutionary units, and to investigate the evolutionary times and processes involved in their diversification.

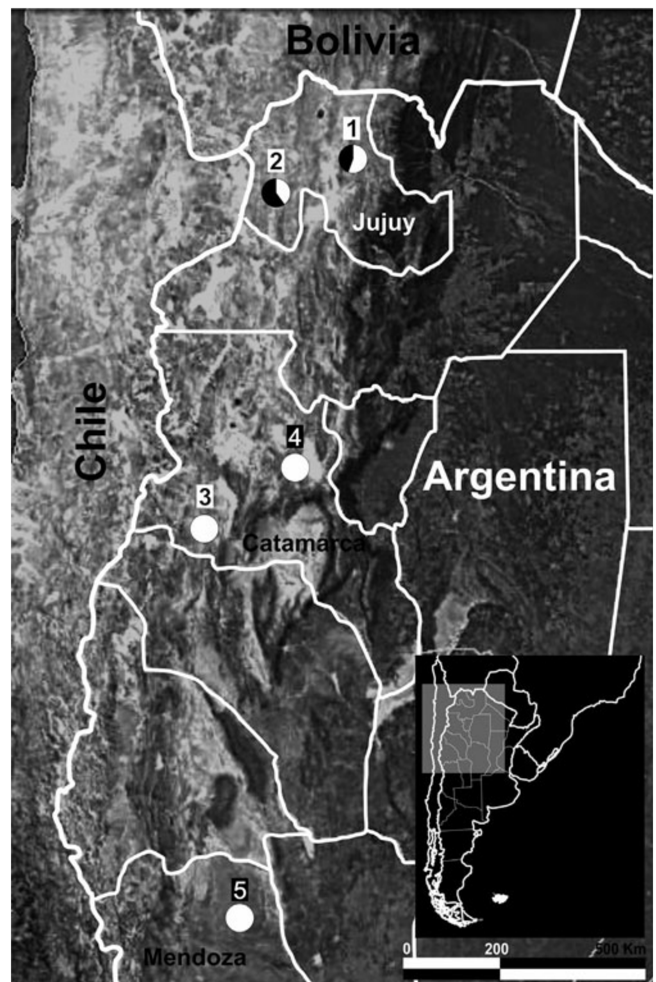

Fig. 1. Map of Argentina showing the populations of *Eligmodontia puerulus* (1–3) and *E. moreni* (4–5) studied here. In the circles are indicate the quantitative ratio of haplogroups found in each population. The haplogroup exclusive of *E. puerulus* is show in black, and that shared by both species in white.

Material and methods

Population sites, sample collection, DNA extraction and sequencing

Thirty seven individuals of *E. puerulus* and 23 of *E. moreni* belonging to five localities and with different diploid number (2n) were analyzed for the mtDNA control region and flanking sequences (Fig. 1; Table S1). On the other hand, for the IRBP gene, we used 14 selected individuals of both species (Table S1). All sequences were deposited in GenBank (Table S1). Localities and number of individuals (N) are as follows: Abra Pampa (N = 18), Jujuy Province; 26 km W of Susques (N = 10), Jujuy Province; Cortaderas (N = 9), Catamarca Province; Campo Arenal, Los Nacimientos (N = 9), Catamarca Province; Telteca Reserve (N = 14), Mendoza Province (Fig. 1). The first three populations correspond to the Puna desert biome where *E. puerulus* inhabit, and the last two are part of the Monte desert biome where *E. moreni* were collected. Coordinates (longitude/latitude/altitude), accession numbers of GenBank, and additional data are showed in Table S1. Voucher specimens are housed in the mammal collection of the Instituto Argentino de Zonas Aridas (CMI – IADIZA), CCT-Mendoza, CONICET.

For the molecular analyses, total DNA from muscle or liver tissues were extracted following the protocol for mammalian tissue kit: AccuPrep® Genomic DNA Extraction Kit, with mini-

mal modifications. A fragment of approximately 1250 pb of the mtDNA was amplified by polymerase chain reaction (PCR) using the primers 464 (5′-TGAATTGGAGGACAACCAGT-3′) and 282 (5′-AAGGCTAGGACCAAACCT-3′). Amplification conditions were those described by González-Ittig et al. (2002) with minimal modifications. The reaction started with denaturation at 94 °C (3 min), followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (90 s) and extension at 72 °C (90 s). Finally, there was a hold period at 72 °C (5 min). Additionally, of selected individuals (Table S1), a fragment of the first exon of the IRBP gene was amplified using the primers A1 (5′-ATGCGGAAGGTCCTCTTGGATAAC-3′) and F1 (5′-CTCCACTGCCCTCCCATGTCT-3′) by means of a touchdown protocol with four stages (Jansa and Voss, 2000). The first stage consisted of five cycles of denaturation at 95 °C for 20 s, annealing at 58° C for 15 s and extension at 72 °C for 60 s. For the second and third stages the annealing temperature was changed to 56 and 54 °C, respectively. The final stage consisted of 23 cycles at 52 °C of annealing temperature. Amplification reactions started with a DNA denaturation at 95 °C for 10 min and ended with a DNA extension at 72 °C for 5 min. Negative controls were included in all experiments. PCR products were purified following the protocol for purification of PCR products: PCR Purification Kit AccuPrep®, and sequenced in both directions. Sequence alignment was carried out with the MEGA 6.0 (Tamura et al., 2013) and corroborated by eye inspection.

Population genetics

We calculated the genetic distances (K2P) for all pairwise sequences with MEGA 6.0. After that, the mitochondrial sequences were collapsed into haplotypes in the DNASP 5.1 software (Librado and Rozas, 2009). Sequence diversity statistics were calculated for each *Eligmodontia* species and population. Summary statistics included number of haplotypes, and nucleotide and haplotype diversity indices. These parameters were estimated using DNASP 5.1 software (Librado and Rozas, 2009) and Arlequin 3.5 (Excoffier and Lischer, 2010). To investigate the demographic history, we examine possible evidence for population growth in the past by Tajima' D and Fu's Fs indexes and its corresponding *p*-values using 1000 simulations with Arlequin 3.5.

An Analysis of Molecular Variance (AMOVA) was performed to examine the intraspecific hierarchical genetic structure (Excoffier et al., 1992). Populations used for this analysis were defined a priori based on their geographic distribution without a genetic criterion. The global F_{ST} index was calculated from AMOVA. Statistical significance was test using 1023 permutations. Genetic differentiation between populations was estimated by pairwise F_{ST} comparisons. These analyses were performed using the Arlequin 3.5 software.

The number of genetic groups or clusters (*K*) was estimated by means of Bayesian theory. Once *K* was determined, the total number of individuals was probabilistically assigned to these clusters. Bayesian analysis was performed using a mixture model for linked loci due to the non-recombinant nature of mtDNA. Simulations were run ten times for $K = [1–5]$. The size of ten-best visited partitions with their log marginal likelihood [log (ml)] values is used to estimate the "correct" number of clusters (Corander et al., 2006). The partition with the highest log (ml) value was considered as the optimal cluster number.

The evolutionary relationships among haplotypes, for the mitochondrial sequences, were explored using the Network 4.6 software (Bandelt et al., 1999). A median network containing all possible maximum parsimony trees was constructed. We used the Median Joining algorithm and a value of zero for the epsilon parameter. All characters were treated with equal weight.

Evolutionary scenarios

We used DIYABC v2.1.0 software (Cornuet et al., 2014) to compare competing hypotheses about the origin of genetic diversity in the *E. puerulus* – *E. moreni* ingroup. The hypotheses were constructed primarily to test the time of divergence in the ingroup, as well as the possibility of species admixture after initial divergence (Fig. S1). The groups were defined based on population genetics results together with geographical origin of samples as follows: (1) P1 (*E. puerulus* samples from Jujuy Province, N = 28), (2) M (all *E. moreni* samples, N = 23), and (3) P2 (*E. puerulus* samples from Catamarca Province, N = 9).

Table S2 shows the prior definitions and distributions for all hypothetical scenarios. The prior definition of the time of divergence between *E. puerulus* and *E. moreni*, and prior distribution of mutation rate, were established from different approaches. First we considered that the general level of sequence divergence for the entire mtDNA molecule in mammals averages 2% per million years ($\mu = 2 \times 10^{-8}$) (Brown et al., 1979). We also considered that the medium time of divergence obtained between *Calomys* and *Eligmodontia*, in a molecular phylogenetic analysis with cytochrome b and three calibrations based on the fossil record, was 7 Ma (Parada et al., 2013). The genetic distances between both genera in our data set (which is based on a different molecular marker) range from 19 to 25%. This produced a range of 2.7–3.6% of divergence per Ma ($\mu = [2.7 \times 10^{-8}–3.6 \times 10^{-8}]$) for mitochondrial control region and flanking sequences. Considering these estimations, we used a range of mutation rate $\mu = [1 \times 10^{-9}–1 \times 10^{-7}]$ because of it includes both values. Before the final analyses, we run several trials to adjust the prior distributions in order to accommodate a wide range of effective population sizes and divergence times. Split times were translated into years by assuming a generation time of 1 year (Pearson et al., 1987). For the initial divergence a wide range of time was used (from 0 to 1,500,000 YBP). The upper limit considers a mutational rate of 2% per million years, which is the lowest mutation rate estimated for mitochondrial markers under this study in the biological model. Also we set a posterior split at between 0 and 1,200,000. Finally, an admixture event occurred between 0 and 500,000 was tested, and the upper limit considers the maximum divergence of P2 compared with M (0.96%). The population effective sizes were set as NA > NP1; NA > NM and NA > NP2 (Table S2). The parameters were simulated using a K2P model with 10% of the invariant sites and a gamma shape of 2. Summary statistic considered where: number of haplotypes and mean number of pairwise differences within and among groups, totaling 12 summary statistics. We simulated four million data sets for the comparison between the four scenarios (~one million each). To determine the most likely scenario, the normalized Euclidean distances between each simulated dataset of the reference table and the observed dataset was then computed and 1% of the closest simulated datasets were used to estimate the relative posterior probability (with 95% confidence intervals) of each scenario with a logistic regression. The most likely scenario was the one with the highest posterior probability value and non-overlapping 95% confidence intervals. Once the most likely scenario was assessed, we used a local linear regression to estimate the posterior distributions of parameters. We chose the 1% of simulated data sets closest to our real data set for the logistic regression after applying a logit transformation to the parameter values. In order to evaluate the goodness-of-fit of the estimation procedure, we performed a model checking computation by generating 1000 pseudo-observed datasets with parameters values drawn from the posterior distribution given the most likely scenario (Tables S2–S4).

Phylogenetic analyses

The phylogenetic relationships among samples, for mtDNA control region and flanking sequences dataset, and for IRBP fragment dataset, were estimated using minimum evolution (ME), maximum likelihood (ML) and maximum parsimony (MP). Both groups of sequences (mitochondrial and nuclear fragments) were analyzed separately. MP was performed with TNT (Goloboff and Catalano, 2016), whereas the ME and ML were performed with MEGA 6.0 software (Tamura et al., 2013). The same fragment of the mtDNA of one *E. typus* and one *E. morgani* specimens (Table S1) was also sequenced to be used as outgroups, together with GenBank sequences of the related species *Calomys callidus* (accession DQ926660), *Calomys venustus* (accession DQ926661) and *Calomys laucha* (accession DQ926662). For IRBP analysis, were acquired sequences from GenBank of the *E. typus* (accession AY277445), *Calomys laucha* (accession JQ434404), *Calomys lepidus* (accession KC953361) and *Calomys venustus* (accession KC953362) to be used as outgroups.

ME tree was performed based on K2P genetic distances. MP trees were inferred with 1000 replicates of heuristic search, saving 100 trees per replica, with tree-bisection-reconnection (TBR) branch swapping and random addition sequence. Variable nucleotide positions were equally weighted and treated as unordered characters. In the mitochondrial dataset, the gaps were treated as a fifth character state in the MP analysis. No gaps were present in the IRBP dataset. Then we summarized the trees of MP in a strict consensus tree. For mitochondrial dataset, the model selected by AIC (Akaike Information Criterion) was HKY + G (−ln L = 4004.62) with the following parameters: gamma distribution shape parameter = 0.35; assumed base frequencies A = 0.307, C = 0.223, G = 0.133, T = 0.337; substitution matrix: (A–T) = 0.060, (A–C) = 0.040, (A–G) = 0.090, (T – A) = 0.050, (T – C) = 0.14, (T – G) = 0.020, (C – A) = 0.050, (C–T) = 0.220, (C–G) = 0.020, (G – A) = 0.20, (G–T) = 0.06, (G – C) = 0.04. For nuclear IRBP dataset the model selected by AIC was K2 (−ln L = 1231.75) with the following parameters: assumed base frequencies A = 0.25, C = 0.25, G = 0.25, T = 0.25; substitution matrix: (A–T) = 0.030, (A–C) = 0.030, (A–G) = 0.190, (T – A) = 0.030, (T – C) = 0.19, (T – G) = 0.030, (C – A) = 0.030, (C–T) = 0.190, (C–G) = 0.030, (G – A) = 0.190, (G–T) = 0.03, (G – C) = 0.03. The node support was assessed with 1000 bootstrap replicates in the ME and MP analyses and with 500 bootstrap replicates in the ML. Branches with <50% support were allowed to collapse.

Results

Sequence variability

The mitochondrial sequences of *E. puerulus* and *E. moreni* analyzed (ingroup) had 1161pb and include a few bases of the cytochrome b, the tRNAThr, tRNAPro, tRNAPhe and the complete control region. These mtDNA fragments contained 53 variable sites and seven *indels* in regions with repetitive base pairs, which were excluded from the population analyses. The nucleotide frequencies were A = 30.73, T = 33.75, C = 22.20, G = 13.32. There were two groups of pairwise genetic distances K2P among these sequences. One range from 0 to1.05% and the other from 1.85% to 2.93%; there were not intermediate distances (Fig. S2).

The nuclear IRBP exon analyzed in the ingroup had 759 pb and presented very low variability. There was only one fixed substitution that differentiated *E. moreni* from *E. puerulus* samples, and each species had one exclusive polymorphic site, sharing another one. The nucleotide frequencies were A = 23.23, T = 21.50, C = 27.32, G = 27.95.

Population genetic analyses

From 60 samples of the mtDNA analyzed there were a total of 44 haplotypes excluding gaps, and 47 haplotypes including gaps. No haplotype was shared by the two nominal species (Table S1). The haplotype and nucleotide diversity were high in all species and populations, with evidence of expansion either when considering the totality of samples or when restricting the analysis to the samples of *E. moreni* (Table 1). However, when each population was analyzed separately, the data did not deviate from the expected for populations with constant size that evolve neutrally (Table 1).

In *E. puerulus* approximately 92% of the mitochondrial genetic variation was detected within populations. The remaining variation was distributed among populations (∼8%). In *E. moreni* approximately 90% of the mitochondrial genetic variation was detected within populations and the remaining variation was distributed between populations (∼10%). Global F_{ST} were statistically significant in both species ($p < 0.05$), for *E. puerulus* $F_{ST} = 0.077$ and for *E. moreni* $F_{ST} = 0.097$. According to Wright's qualitative guidelines for the interpretation of F_{ST} (Wright, 1978), the populations of both species were moderately structured. For *E. puerulus* pairwise F_{ST} comparison between Susques (Jujuy Province) and Cortaderas (Catamarca Province), and between Abra Pamapa (Jujuy Province) and Cortaderas were statistically significant ($p < 0.05$) $F_{ST} = 0.027$ and $F_{ST} = 0.104$, respectively. These results indicate little and moderate genetic structure between these population pairs, but not between populations from Jujuy.

Three clusters were defined under the Bayesian model [log (ml) = 0.998]. The 23% of *E. puerulus* specimens (N = 14) were assigned to cluster 1, 23% of individuals (N = 14) from both species were assigned to cluster 2, whereas 54% of individuals (N = 32) from both species were assigned to cluster 3 (Fig. 2).

The network showed two major separate groups, distanced by 20 substitutions: one included samples of *E. puerulus* from Jujuy Province exclusively (haplogroup A), and the other included samples from all studied localities (haplogroup B, showed in Fig. 2). All sequences of *E. puerulus* from Cortaderas (Catamarca Province) were included in the major haplogroup that also contains the totality of *E. moreni*ís samples. Four sequences of *E. puerulus* from Susques (4/10), and ten of *E. puerulus* from Abra Pampa (10/18) also fall into this major haplogroup B. These sequences of *E. puerulus* were joined to the network in three sub-groups. One included sequences of *E. puerulus* from the three sampled localities, and the other included sequences exclusively from Cortaderas. These two sub-groups were connected to the same hypothetic haplotype in one extreme of the network, which also joined another haplotype from Cortaderas. Additionally, two haplotypes of *E. puerulus* from Cortaderas were mixed with *E. moreni*ís samples in cluster 2, close to the join of both major haplogroups (Fig. 2).

Phylogenetic analyses

Some of the new generated sequences (Table S1) were obtained from the same individuals used in Lanzone et al. (2011), where the cytochrome b variation was analyzed, but with a lower sample size (12 specimens with known chromosome complements: 3 *E. moreni* and 9 *E. puerulus*). So we compared their phylogenetic position. As expected from linked genetic markers, different sequences from the same individual have an identical pattern of join in the tree (data not shown).

For mitochondrial dataset, all phylogenetic analyses (ME, MP and ML) generated similar tree topologies, where *E. puerulus* and *E. moreni* were not recovered as reciprocally monophyletic, but as polyphyletic. The most basal dichotomy of this clade included an exclusive group of *E. puerulus* sequences from Jujuy with high statistical support in the three analyses, and another clade grouping

Table 1
Statistical data of genetic diversity assessed for *Eligmodontia moreni* and *E. puerulus* samples using the mitochondrial sequences.

Group	N	H/Hd	Pi	S	K	D	Fs
All samples	60	44/0.978	0.01178	53	13.592	0.662 NS	−18.987***
All *E. puerulus*	37	26/0.950	0.01328	39	15.342	2.282 NS	−4.425 NS
All *E. moreni*	23	18/0.976	0.00582	28	6.727	−0.429 NS	−7.532**
E. moreni Mendoza	14	11/0.956	0.00531	18	6.143	0.354NS	−3.041 NS
E. moreni Catamarca	9	7/0.944	0.00629	17	7.278	0.407 NS	−0.543 NS
E. puerulus Caramarca	9	7/0.944	0.00418	12	4.83333	0.449 NS	−1.311 NS
E. puerulus Abra Pampa, Jujuy	18	11/0.856	0.01364	34	15.765	2.407 NS	1.593 NS
E. puerulus Susques, Jujuy	10	10/1	0.01465	33	16.933	2.177 NS	−2.156 NS

N, Number of individuals examined; H, number of haplotypes; Hd, haplotype diversity; Pi, nucleotide diversity; S, number of segregating sites; k, average number of pairwise nucleotide differences. Tajima's D, and Fu's Fs statistics for the *Eligmodontia* species and populations is presented in the last columns. Statistical significance for Tajima's D and Fu's Fs statistics: <0.005**; <0.0001***.

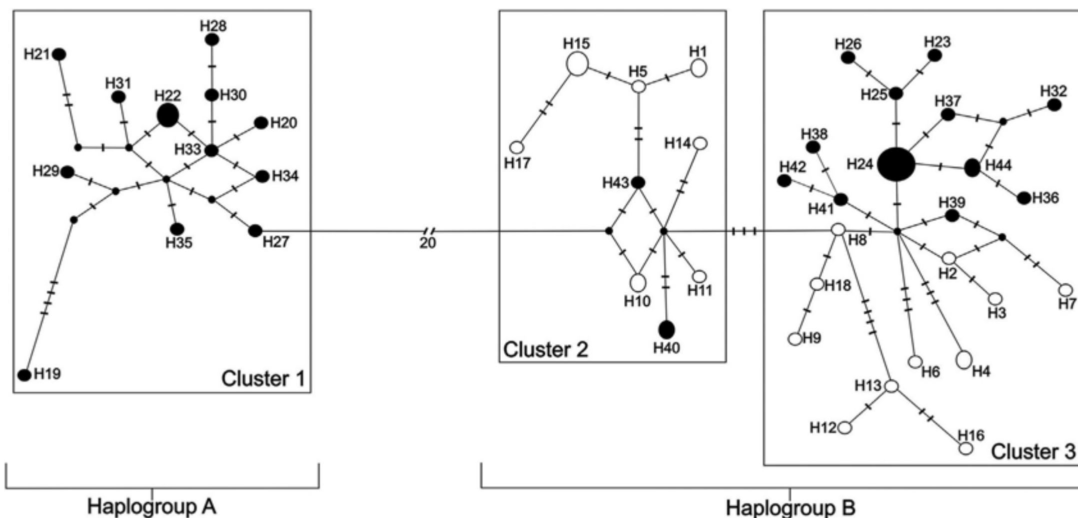

Fig. 2. Median network of *E puerulus* (black circles) and *E. moreni* (white circles) haplotypes. The size of the circles corresponds to the haplotype frequencies. The mutational steps between haplotypes are represented by small perpendicular lines. Small dots indicate hypothetical haplotypes. See Table S1 for the haplotype designations. In boxes are indicated the clusters recovered by the Bayesian analysis.

sequences of all populations sampled of *E. puerulus* and *E. moreni*, also with high support (Fig. 3A). The MP analysis of mitochondrial dataset has 335 variable sites, 30 *indels* and 283 parsimony informative characters. This analysis generated 40 most parsimonious trees (length = 511, consistence index CI = 0.80, retention index RI = 0.90). Sequences of both nominal taxa were grouped separately in different subclades with low to median bootstrap support. The only close interspecific join was observed between a sequence belonging to *E. moreni* from Campo Arenal (Catamarca Province) and another corresponding to a specimen of *E. puerulus* from Cortaderas (Catamarca Province). This grouping had low support in all phylogenetic analyses (Fig. 3A).

The dataset of the IRBP nuclear fragments (759pb) considered in the MP analyses showed 25 variable characters, of which 17 were parsimony informative. This analysis recovered one most parsimonious tree (length = 27, CI = 0.93, RI = 0.96), which showed a similar topology to those obtained in the ME and ML analyzes (Fig. 3B). All sequences of *E. puerulus* were grouped in a monophyletic clade with moderated bootstrap support and that of *E. moreni* collapsed in a basal polytomy.

Evolutionary scenarios

In the ABC analysis all four scenarios had similar low posterior probabilities, although Scenario 4 was the most supported (Fig. S1, Table S3). The scenario 4 describes a first event of population divergence occurring between P1 (*E. puerulus* samples from Jujuy Province) and M (all *E. moreni* samples), and a second split of P2 (*E. puerulus* samples from Catamarca province) from M. For this scenario, none of the observed summary statistics deviate significantly from the simulated distributions ($p > 0.05$) according to model checking computation analysis.

The parameter estimates for this scenario are given in Table S4. The divergence between *E. puerulus* and *E. moreni* occurred approximately 1040000 YBP, near the end of early Pleistocene, whereas the split of P2 group occurred approximately 505000 YBP at the middle Pleistocene. The current population effective sizes for groups were inferred as NP1 > NP2 and NM > NP2. Also, calculations from current populations effective sizes were lower than the ancestral population size, where NA ∼ 3 times higher than NP1 and NM, and NA ∼ 6 times higher than NP2.

Discussion

Species concepts and their application has been the subject of extensive work and debate (Baker and Bradley, 2006). In *Eligmodontia*, traditional taxonomy based principally on morphology, led to different interpretations of species number. From the original description of seven species and several subspecies, the genus suffers a posterior clustering in one species with two subspecies (Hershkovitz, 1962 and literature cited there). The recent integrative taxonomy approach, based on molecular, chromosomal, and morphological evidence, included seven species (five from the original descriptions and two new species) with no subspecies.

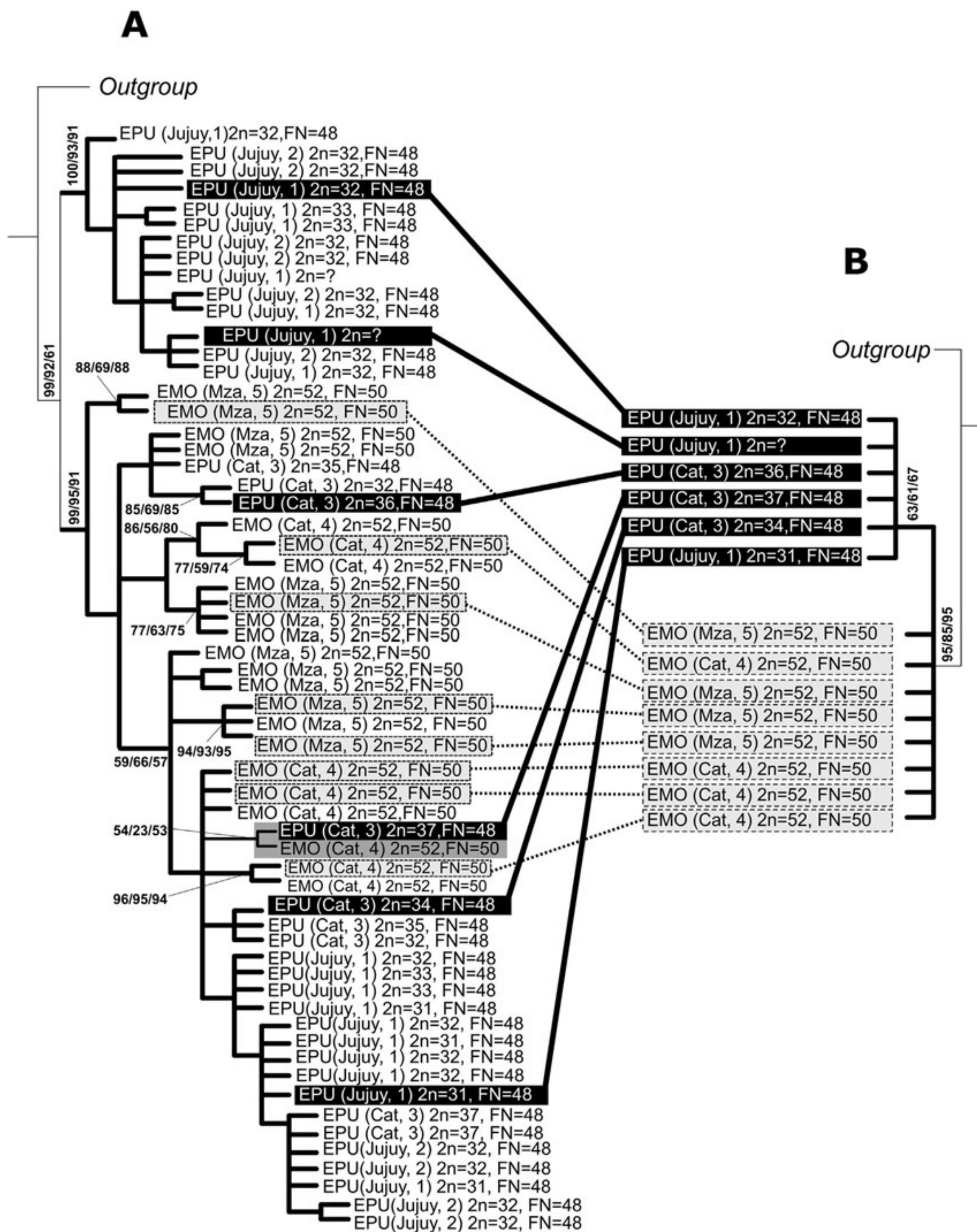

Fig. 3. Phylogenetic trees of *E. puerulus* (EPU) and *E. moreni* (EMO) obtained with the mitochondrial sequences (A) and with the IRPB nuclear exon sequences (B) by MP criterion. The numbers close to the branches are bootstrap support for the nodes in the ME/MP/ML analyses, respectively. Bootstraps lower than 50% are not shown. Cat = Catamarca Province; Jujuy Province and Mza = Mendoza Province. The numbers of the populations are the same as in figure 1. The 2n and FN of each specimen is indicated next to each terminal. Lines joining the terminals of both trees indicate same specimen.

However two of them are polyphyletic at molecular level, which contradicts current basic genetic species concepts. The relatively low genetic distances and the absence of reciprocal monophyly between *E. puerulus* and *E. moreni* could be arguments which dispute the validity of one of them. In contrast, the integrative taxonomy approach indicates that the chromosomal and morphological differentiations are large enough to maintain both as independent evolutionary units, and thus supporting their species status (Lanzone et al., 2011).

Based on cytological information, *E. puerulus* (2n = 31–37, FN = 48) and *E. moreni* (2n = 52, FN = 50) differ by several chromosome changes. As deduced from conventional karyotypes (the highest 2n found to date in *E. puerulus*, FN, and 2n/FN of *E. moreni*) at least seven chromosomal rearrangements (and at least one non-Rb) are necessary to acquire this differentiation. If non intermediate 2n exists, the minimal deduced differences are large enough to infer reproductive isolation at meiotic level, and sustain speciation (King, 1993). Besides that banding techniques are needed to fully understand the chromosome differences, the observed modification in FN between *E. moreni* and *E. puerulus* can be the result of one tandem chromosome fission. This chromosome rearrangement has been described as fixed differences between species in other sigmod-

ontine rodents, but not as polymorphisms (Elder, 1980; Spotorno et al., 2001 and literature cited therein). Such chromosome changes have important effects, and are expected to lead to reproductive isolation between differentiated populations (King 1993; Taylor, 2000).

In the phylogeny of the genus, *E. puerulus* and *E. moreni* are included in a major clade that also groups *E. hirtipes* and *E. dunaris* (Spotorno et al., 2013). This group of four species shares a northern distribution and high FNs. Except *E. moreni*, the others species have a FN = 48 (Spotorno et al., 2013). G-bands studies showed that the chromosome differences between *E. puerulus* (2n = 34, FN = 48) and *E. hirtipes* (2n = 50, FN = 48) included seven Rb fusion, one inversion, and a chromosome without evident homology (Spotorno et al., 1994). This suggests that chromosome differences between *E. puerulus* and *E. moreni* may be greater than those inferred by conventional karyotypes. The presence of 2n = 50, FN = 48 also in *E. dunaris* indicates that the FN = 48 could be the ancestral condition for the northern *Eligmodontia* clade. Therefore, the increase of FN in *E. moreni* is a derived condition, which is supported by its derived position in all molecular phylogenetic analyses (Mares et al., 2008; Lanzone et al., 2011; Spotorno et al., 2013).

At the molecular level, we found two major haplogroups, with no intermediate variants, indicating an isolation period that facilitated the divergence of haplotype groups. However, the distribution pattern of the observed mitochondrial variability was not coincident with the previously delimited species through karyotypic and morphologic characters. This incongruence can be explained by several evolutionary processes, as gene flow after isolation, or by a recent divergence between the species that produce incomplete lineage sorting. Although in some case it is very difficult to distinguish between these processes, different sources of evidence suggest the occurrence of one of them (Maddison, 1997; Funk and Omland, 2003).

Combined evidences of mitochondrial and nuclear data confirm the recent divergence of these populations (Lanzone et al., 2011; this work). Additionally, the geographic localization and the position in the phylogenetic tree of mixed genomes (inferred from mitochondrial versus chromosome data) are indicative of actual or ancient hybridization (Funk and Omland, 2003). In the samples of *Eligmodontia* studied here, the only close inter-specific join in the phylogenetic tree correspond to one specimen of *E. moreni* from Campo Arenal in Catamarca Province, and one of *E. puerulus* with 2n = 37 from Cortaderas, also in Catamarca. Interesting, this is the highest 2n found in *E. puerulus*, which is the expected to produce lower hybrids infertility in inter-specific crosses, because involves minor chromosome differences (at least seven chromosomes changes) between mating individuals of both species. In this polymorphic population of *E. puerulus* from Catamarca, all mitochondrial sequences were more similar to that of *E. moreni*, suggesting the present or past existence of sympatric zones with hybridization in Catamarca, Argentina. Among these species never were detected sympatry nor hybrid individuals (Lanzone et al., 2007, 2011; Mares et al., 2008). However, the data showed that in some cases the contact zones between hybridizing species are very narrow (Coyner et al., 2015), indicating that in order to discard current hybridization additional areas can be investigated. Hybridization is a very poorly documented phenomenon in sigmodontine rodents (Patton et al., 2015), and this is the first clear molecular documentation of introgression previously suspected for *Eligmodontia* (Lanzone et al., 2011).

Another indicative of the evolutionary process is the position of the supposed hybrids in the network. In the case studied here, there are two major different groups of introgressant haplotypes. One group of few samples was located very close to the connection of the two major haplogroups. The other group was connected at the end of the network, in two separated subgroups, joining to an hypothetical haplotype to which also are join others sequences of *E. moreni*. This pattern could be due to an ancient sporadic and recurrent hybridization, involving few individuals (Melnikova et al., 2012). It was observed that in some case speciation process can occur with intermittent limited genetic interchange (Baker and Bradley, 2006), which could be the case of *E. puerulus* and *E. moreni*.

One interesting result from this study is that only *E. puerulus* included both haplotype groups. This suggests that introgression occurred from *E. moreni* to *E. puerulus* and not in the reciprocal crosses. Asymmetrical introgressive hybridization was observed in other taxa (Patton and Smith, 1994; Abramson et al., 2009; Melnikova et al., 2012; Coyner et al., 2015), in some of which the complete replacement of the mitochondrial genome of one species with that of the other was reported (Melo-Ferreira et al., 2012). In the southern populations of *E. puerulus* (Catamarca), the replacement of an original *E. puerulus* mtDNA genome by that from *E. moreni* is complete. But this is not the situation in the northern populations of Jujuy Province where both haplogroups were found in high frequency.

Nuclear sequences indicated no introgression, but the low variability in the IRPB, the unique nuclear gene tested, and the low sample size analyzed demonstrated that additional data are needed to know the extent of nuclear genome isolations of both taxa. The studies of hybrid zone show that introgression of distinctive molecular data (mitochondrial sequences, autosomal sequence, linked genes to sex chromosomes, microsatellites data) have different geographical extensions in the same region (Beysard and Heckel, 2014; Coyner et al., 2015).

E. puerulus is distributed in the Puna desert, an old and heterogeneous landscape composed of valleys surrounded by high mountains and volcanoes (Allmendinger et al., 1997). This region has intense tectonic activity since more than ten million years, which probably impacted in the distribution of genetic variability of this species. This is especially important to interpret the high chromosomal variability and differentiation among populations of *E. puerulus* (Lanzone et al., 2011), which could be favored by the complete or partial isolation of valleys where the species inhabit. This could also have favored the high genetic diversity maintained in these populations, as indicated for the high haplotype and nucleotide diversity observed. Additionally, genetics data showed a third group of coalescent sequences shared between both species, indicating a multiple origin of the genetic variability of *E. puerulus* and *E moreni*.

On the other hand, the contiguous area to the east where *E. moreni* inhabit is a relatively young landscape, known as region of the Sierras Pampeanas. The uprising of these Sierras dates from the middle and late Pliocene, a time of intense Andean orogeny that culminates with the formation of Precordillera and the semi-desert of shadows of the western region of Argentina, currently known as Northern Monte desert (Ortiz-Jaureguizar and Cladera, 2006). The species mainly living in that region at present (*E. moreni* and *E. bolsonensis*) are the species involved in the more recent divergences in the genus (Lanzone et al., 2007, 2011; Mares et al., 2008). Interestingly, this geographical region has also been pointed out as the landscape where the split of other species of phyllotine rodents belonging to genus *Graomys* has occurred. *G. griseoflavus* and *G. chacoensis* are distributed in the Monte desert and Chaco respectively. These species are characterized by low molecular divergence (Theiler et al., 1999; Martínez et al., 2010), and karyotypes that differ in several chromosomes changes (Lanzone et al., 2014), indicating that this region has an important role in promoting the divergence of some taxa. In addition, the split of the northern populations of *E. puerulus* and the populations of *E. moreni* was dated near the end of early Pleistocene, and the origin of the southern population of *E. puerulus* in Catamarca (that which shared the haplotypes variant also found in *E. moreni*) was dated in middle

Pleistocene. Most of Peistocene (from 1,810,000 to 10,000 YBP) was characterized by cyclic climatic changes that expand and contract the distribution areas of taxa, communities, and biomes (Ortiz-Jaureguizar and Cladera, 2006). These climatic oscillations possibly had a major influence in the isolation and subsequent contact of *Eligmodontia* populations, suggesting that geologic and climatic changes promoted the patterns of genetic variations observed in these rodents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2017.06.001.

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