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MOLECULAR AND PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL CONTROL REGION IN ROBERTSONIAN KARYOMORPHS OF *GRAOMYS GRISEOFLAVUS* (RODENTIA, SIGMODONTINAE)

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ABSTRACT: The South American rodent *Graomys griseoflavus* is a species with a Robertsonian (Rb) autosomal polymorphism. A marked genetic differentiation between 2n=42-41 and 2n=38-34 karyomorphic groups was evidenced by cytogenetic and molecular analysis. The mitochondrial control region was sequenced in all *Graomys* karyomorphs for its characterization and used to trace more accurate phylogenetic relationships. The molecular organization showed to be coincident with the consensus molecular structure described for other rodent taxa, exhibiting the conserved domains ETAS (extended termination-associated sequences), CD (central domain) and CSB (conserved sequence block) 1, 2 and 3. Phylogenetic trees showed that 2n=42-41 and 2n=38-34 karyomorphic groups form separate clades, with neither phylogeographical structure nor population subdivision within Rb karyomorphs. These findings suggest a short evolutionary time for the occurrence and fixation of the chromosomal rearrangements and reinforce the single origin hypothesis for the Rb karyomorphs of *G. griseoflavus*.

Key words. Rodentia. *Graomys*. Robertsonian polymorphism. mtDNA. Phylogeny.

INTRODUCTION

Graomys griseoflavus Waterhouse 1837, is a sigmodontine rodent with an ample Robertsonian (Rb) autosomal polymorphism, showing karyomorphs with diploid numbers equal to 42, 41, 38, 37, 36, 35 and 34 (Zambelli et al., 1994, 2003). Cytogenetic, molecular, and reproductive data support the ancestry of the 2n=42 karyomorph (Gardner and Patton, 1976; Zambelli et al., 1994; Theiler and Blanco, 1996 a, b; Zambelli and Vidal-Rioja, 1999) from which two lines have derived: one producing very low frequent 2n=41 individuals, and the other, giving rise to 2n=38 specimens. These latter have appeared as a consequence of two homozygous (Hm) Rb fusions (RF): RF15-17 and RF16-18 that reduced the diploid number. Thereafter, starting from the 2n=38, the 2n=37-34 karyomorphs were derived by a non-random downward sequence of Rb fusions: RF1-6 and RF2-5 (Zambelli et al., 1994). There are no significant geographic barriers separating different populations of *Graomys* (Theiler and Blanco, 1996a,b).

Molecular-cytogenetic analysis of *G. griseoflavus* chromosome evolution showed a marked differentiation between 2n=42-41 and 2n=38-34 karyomorphic groups. Based on allozyme patterns and reproductive behavior (Theiler and Blanco, 1996 b; Theiler et al.,

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1999) the taxonomic status of *G. griseoflavus* was revised reassigning them to *Graomys centralis*, for 2n=42 specimens, and *Graomys griseoflavus*, for those of the 2n=38-36 complex. However, the authors did not include in the revision 2n=41, 35, and 34 individuals. The genetic differentiation observed with nuclear DNA markers and the breeding tests, support that 2n=42-41 and 2n=38-34 constitute at least two sibling species.

HmRF15-17 and HmRF16-18 have arisen as a chromosomal feature common to the 2n=38-34 complex; moreover, their occurrence may be correlated with NOR pattern and satellite DNA organization (Zambelli and Vidal-Rioja, 1996; Zambelli and Vidal-Rioja, 1999; Zambelli et al., 2003).

The parsimony trees obtained comparing mitochondrial cytochrome *b* (cyt *b*) sequences from all *Graomys* karyomorphs showed Rb karyomorphs (2n=38-34 complex) grouped in a single clade, while the ancestral 2n=42 animals and the 2n=41 karyomorph formed a different one. This agrees with the karyomorphic differentiation evidenced by nuclear markers and is consistent with the hypothesis of a single origin for Rb karyomorphs (Catanesi et al., 2002). The analysis of cyt *b* sequence of *G. griseoflavus* contributed to bring light onto the origin of the Rb karyomorphs, however one aspect still not clarified is the phylogenetic relationships among the 2n=38-34 derived karyomorphs, particularly among the 2n=38, 37 and 36 karyomorphs and the RF2-5-carrying 2n=35 and 34 karyomorphs. With this purpose in the present work we sequenced in *Graomys* the main non-coding region of the mitochondrial genome: the displacement loop (D-loop) or control region. The evolution of the control region of mammalian mtDNA shows some features such as strong rate heterogeneity among sites, the presence of tandem repeated elements, a high frequency of nucleotides insertion/deletion, and lineage specificity (Pesole et al., 1999; Larizza et al., 2002). This region contains the origin of mtDNA replication, and therefore, it is a triple strand structure (Randi et al., 1998; Larizza et al., 2002). Typical mammalian control region shows three domains: extended termination-associated sequence (ETAS, spanning from the $tRNA^{Pro}$ gene to the central domain); the central domain (CD); and the conserved sequence block (CSB, from the CD to the $tRNA^{Phe}$ gene) (Sbisà et al., 1997). In mammals, the substitution rate within the control region is not uniform since two peripheral fragments concentrate as much as the 90% of the variation. These two fragments are always flanking the much more conserved CD. Therefore, the peripheral regions are useful in populational studies, while the conserved regions are very informative for reconstructing phylogenies among recently diverged taxa (Arnason et al., 1993; Arctander et al., 1996; Randi et al., 1998; Maté et al., 2004). Moreover, many mammalian control region sequences are currently available, making this region a model for studies of recent mammalian evolution (Sbisà et al., 1997; Matson and Baker, 2001; Larizza et al., 2002).

The aim of this work was to characterize the molecular organization of the control region of all *Graomys* karyomorphs, and investigate the evolutionary dynamics of this region in concordance, if any, with the karyotype rearrangements.

MATERIALS AND METHODS

Collection of specimens

Twenty one specimens of *Graomys* representing all karyomorphs were collected by field trapping in the following localities (**Table 1**): Santiago Temple (31º 23' S, 63º 25' W), and General Belgrano (31º 59' S, 64º 34' W), in Córdoba Province (2n=42); Deán Funes (30º 24' S, 64º 21' W), approximately 150 km northwest from Santiago Temple (2n=41) in Córdoba Province; Salicas (28º 22' S, 67º 03' W), approximately 600 km northwest of Santiago Temple area in La Rioja Province (2n=38, 37, 36); Divisadero Largo (32º 53' S, 68º 51' W), approximately 450 km south from Salicas area and 600 km west from Santiago Temple in Mendoza Province (2n=36, 35, 34); and Los Menucos (40º 51' S, 68º 5' W), approximately 850 km south from Mendoza in Rio Negro Province (2n=34). These individuals were karyotyped (as described in Zambelli et al., 1994) and total DNA

Table 1

Geographic origin, diploid number (2n), number of specimens (No.), and GenBank accession numbers of control region sequences of the analyzed karyomorphs of *Graomys griseoflavus*.

Locality	2n	No.	Accession number
Santiago Temple	42	4	AY357923; AY359285 AY398731; AY398732
General Belgrano	42	5	AY359680; AY364007 AY398733; AY398734 AY398735
Deán Funes	41	1	AY398736
Salicas	38	2	AY359679; AY364006
	37	3	AY398738; AY364008 AY398737
	36	2	AY398740; AY398741
Divisadero Largo	36	1	AY398739
	35	1	AY398742
	34	1	AY398744
Los Menucos	34	1	AY398743

was obtained from fixed liver (as described in Zambelli and Vidal-Rioja, 1995).

mtDNA control region studies

Approximately 1100 bp of mtDNA corresponding to CR and flanking $tRNA^{Pro}$ and $tRNA^{Phe}$, were PCR-amplified using primers from *Mus musculus* (Nachman et al., 1994) L15320 5'-ATAAACATTACTCTGGCTACTTGTAAACC-3', and H00072 5'-ATTAATTATAAGGCCAGGACCAAACCT-3'. PCR cycling was 94°C for 2 min, and then 35 cycles of 94°C for 45 sec, 52°C for 50 sec and 72°C for 60 sec, followed by a final extension of 72°C for 5 min. The PCR fragments were ligated to the PCR-cloning vector pGEMT-Easy vector (Promega) and the ligation mix used to transform XL1Blue *E. coli* strain (Stratagene). Recombinant clones were selected in LB-Amp-Xgal plaques. Manual sequencing of recombinant plasmids was performed by the dideoxy chain termination method using ^{32}P-dATP, and T7 DNA polymerase kit (Pharmacia). Sequencing mixes were run on 6% denaturing polyacrylamide gels. The obtained sequences were submitted to GenBank under the accession numbers detailed in **Table 1**.

After alignment, conserved domains ETAS, CD, and CSBs were identified and compared to the corresponding domains of the control regions from *Mus musculus* (V00711), *Rattus rattus* (X04735), and the sigmodontine rodents *Peromyscus levipes* (AF081489), *Akodon molinae* (AF296268), and *Calomys laucha* (AY033227).

Statistical and phylogenetic analyses

Assuming evolutionary changes determined solely by mutation and random genetic drift and no recombination between DNA sequences, nucleotide diversity was calculated by Kimura-2P distance method by using the Arlequin 2.0 software (Schneider et al., 2000). Differentiation among populations was tested by calculation of pairwise F_{st} values using the same software. The phylogenetic analysis of control region sequences was performed by using PHYLIP package (Felsenstein, 1995). Five hundred bootstrap replicates of the data were obtained with the tool SEQBOOT. Neighbor-joining trees (Saitou and Nei, 1987) were constructed with DNADIST tool, under a maximum likelihood model.

RESULTS

In all *Graomys* karyomorphs the control region was 1082 bp long with minor differences in length when all sequences were aligned (**Fig. 1**). The analysis of the control region sequences allowed the identification of the typical conserved domains and the finding of a consensus molecular organization shared by all *Graomys* karyomorphs studied. Thus, it was

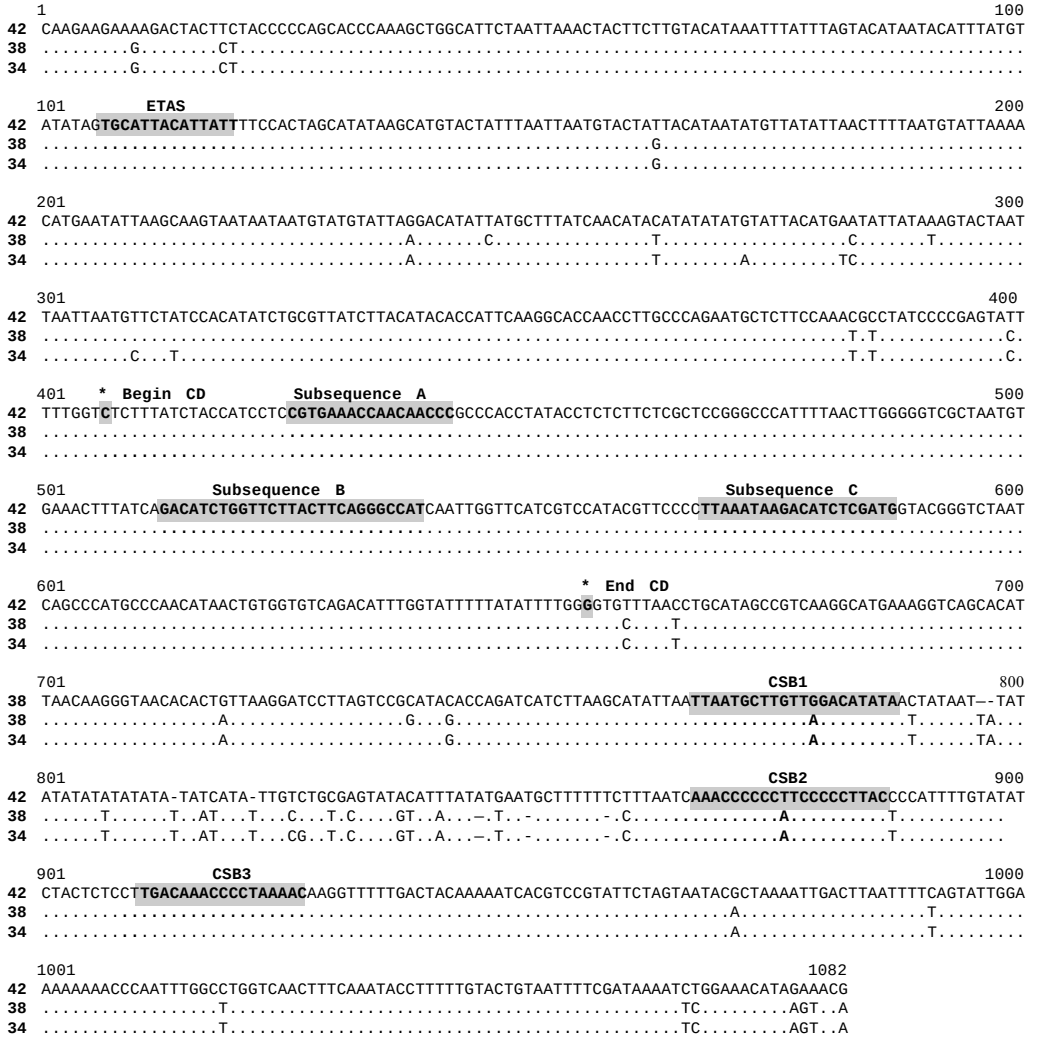


Fig. 1. Alignment of complete control region sequences from three representative *Graomys* karyomorphs with 2n=42, 38 and 34. ETAS, extended termination-associated sequence; CD, central domain; CSB1-3, conserved sequence blocks.

possible to define the domains ETAS, CD, CSB1, CSB2, CSB3 (Walberg and Clayton, 1981; Sbisà et al., 1997; **Fig. 1**). No repeated sequences were found; instead, a $(TA)_n$ dinucleotide microsatellite located between the CSB1 and CSB2 regions was found (**Fig. 1**). In the group 2n=42-41 the microsatellite showed a variable size with 7 to 10 (TA) repetitions. In the Rb 2n=38-34 the microsatellite was imperfect with the sequence $(TA)_5TT(TA)_3T(TA)_1$, and a constant length among all the individuals studied. We analyzed the CR sequence from *Graomys* karyomorphs concentrating on the conserved segments included in ETAS, CSBs and CD (Foran et al., 1988; Gemmell et al., 1996) which were compared to that from muroid related taxa (**Fig. 2**). Thus, alignment of the conserved portion of ETAS showed no differences among *Graomys* karyomorphs and mi-

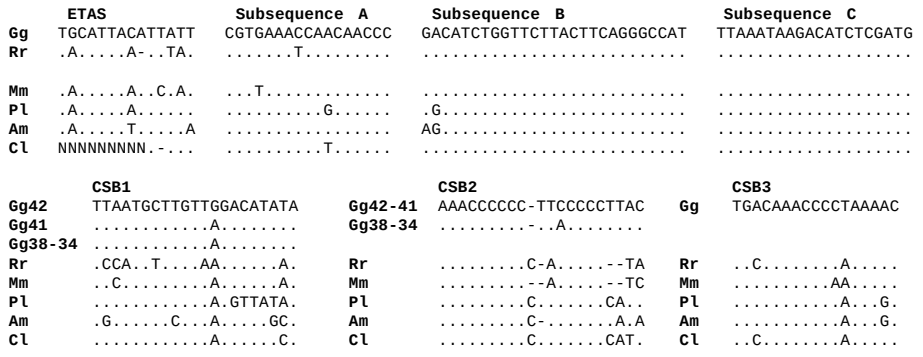


Fig. 2. Alignment of conserved segments of ETAS, subsequence A, B and C (from central domain), and CSB1-3, from *G. griseoflavus* and related rodent species. Gg: *G. griseoflavus*; Rr: *R. rattus*; Mm: *M. musculus*; Pl: *P. levipes levipes*; Am: *A. molinae*; Cl: *C. laucha*.

nor difference when compared to those from other muroid taxa (**Fig. 2**). Regarding CSBs domains, CSB1 and CSB2 showed one nucleotide substitution which differentiates the 2n=42 karyomorphs from the Rb individuals, while CSB3 segment was homogeneous among *Graomys*; comparison to the other rodent taxa showed minor differences among CSBs segments (**Fig. 2**). For the analysis of the CD we took the conserved subsequences A, B, and C (Gemmell et al., 1996). Thus, we found that they were identical among all *Graomys* karyomorphs, with few nucleotide changes with respect to the other rodent taxa (**Fig. 2**).

Control region showed an average sequence conservation of 96.90% within *Graomys* 2n=42-41, 91.02% within 2n=38-34, and 85.14% between these karyomorphic groups. Although Rb 2n=38-34 exhibited a higher number of substitutions with respect to the ancestral 2n=42-41 individuals, nucleotide diversity comparisons showed no significant differences among them ($p<0.05$; **Table 2**).

Population differentiation among all *Graomys* karyomorphs resulted in $F_{st} = 0.867$ ($p<0.05$) for 100 permutations (**Table 2**). Within 2n=42-41 individuals, F_{st} values were calculated grouping the individuals by geographical location, being all pairwise comparisons equal to zero. For Rb animals, they were grouped both by geographical location and by chromosome number (2n=37-38, 36, and 35-34); all pairwise comparisons gave F_{st} values equal to zero. These results suggest that no population subdivision is present either within 2n=42-41 or Rb animals (**Table 2**). In the light

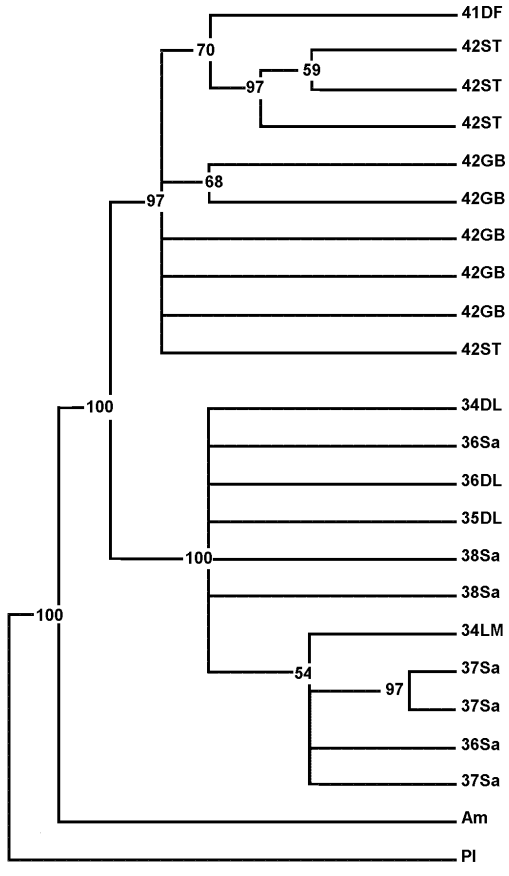
Table 2

Molecular diversity indexes in the 2n=42-41 and Rb 2n=38-34 populations of *G. griseoflavus*.

Index	2n=42-41		2n=38-34
Average sequence homology	96.9%		91.0%
No. of substitutions	21		31
No. of transitions	15		20
No. of transversions	6		11
Nucleotide diversity ($p<0.05$)	0.0052 ± 0.0031		0.0075 ± 0.0043
F_{st} between groups ($p<0.05$)		0.867	
F_{st} within groups ($p<0.05$)	0	0	

of these results we calculated the F_{st} values performing the same pairwise comparison among 2n=42-42 and Rb individuals but analyzing the control region together the cyt *b* sequences previously reported (Catanesi et al., 2002). The F_{st} values within both groups were equal to zero, reinforcing the assumption that no population subdivision is present either within 2n=42-41 or Rb animals.

Phylogenetic analysis was performed by maximum likelihood and the obtained tree revealed two main clades grouping the 2n=42-41 (bootstrap 97%) and the Rb 2n=38-34 karyomorphs (bootstrap 100%; **Fig. 3**). The monophyly of the *Graomys* karyomorphs here analyzed had a good support (bootstrap 100%) respect to *P. levipes levipes* and *A. molinae*. The tree obtained by Jukes-Cantor (1969) method showed identical topology (not shown).



DISCUSSION

The molecular organization of mitochondrial DNA control region from all *Graomys* karyomorphs here reported coincided with the consensus structure described for other rodent taxa, showing the conserved domains ETAS, CD and CSB1, 2 and 3 (**Fig. 1**). Comparative analysis of the complete control region sequences from 23 rodent species revealed as much variability as within mammals (Larizza et al., 2002). For instance, the length of the sequences showed extreme variability, ranging from 878 bp in *M. musculus domesticus* to 1395 bp in *Heliophobius argenteocinereus*. Even members of the same genus (e.g. *Peromyscus attwateri* and *P. boyleri*) or subspecies (*M. musculus musculus* and *M. musculus domesticus*) showed differences both in length and in the presence of repeated elements (Larizza et al., 2002). In some cases length difference is due to the presence of repeated sequences in some species, although the repeats are not the only explaining reason of control region length differences. The specimens of *Graomys* presented in this report did not show significant differences in the whole control region length and, excepting a short dinucleotide microsatellite no other repeated motifs were observed.

The consensus sequences of mitochondrial DNA control region from all *G. griseoflavus* karyomorphs here reported coincided with those described for other rodent taxa, showing the conserved domains ETAS, CD and CSB1, 2 and 3 (**Table 2**). In their study, Sbisà et al. (1997) suggest that though CSB1 is the least conserved sequence block, it is functionally

Fig. 3. Neighbor-joining tree. The numbers at the forks indicate the bootstrap values higher than 50%. Chromosome number and geographic location of each individual analyzed are indicated. Sa: Salicas; DL: Divisadero Largo; GB: General Belgrano; LM: Los Menucos; ST: Santiago Temple; DF: Deán Funes. The rodent species *A. molinae* (Am) and *P. levipes levipes* (Pl) were used as outgroups.

the most important element. This conclusion is based on the observation that CSB1 has been identified in all mammals examined, while CSB2 and CSB3 are sometimes absent. In fact, CSB1 sequences are compatible with functional roles such as the RNA/DNA transition site, the RNAse mitochondrial RNA processing (MRP) cleavage sites, and the 3´end of short RNA primers (Tullo et al., 1995; Larizza et al., 2002).

Within the *Graomys* individuals analyzed, CSB3 was conserved while both CSB1 and CSB2 showed one transition and one transversion, respectively. These substitutions differentiated the ancestral 2n=42 from the Rb 2n=38-34 (**Fig. 2**).

Molecular-cytogenetic analysis of chromosome evolution of *G. griseoflavus* showed a marked differentiation between 2n=42-41 and 2n=38-34 karyomorphic groups. Comparative sequencing of cyt *b* fragments from all *Graomys* karyomorphs allowed to draw parsimony trees showing two well defined clades: one including 2n=42-41 animals (100% bootstrap) and the other with 2n=38-34 individuals (100% bootstrap) (Catanesi et al., 2002), but it was then not possible to clarify the phylogenetic relationships among Rb animals. The present control region analysis allowed to construct trees showing two main clades: 2n=42-41 (bootstrap 97%) and 2n=38-34 (bootstrap 100%; **Fig. 3**). These results were coincident with the obtained previously with cyt *b* sequences (Catanesi et al., 2002) supporting that *G. griseoflavus* karyomorphs constitute a monophyletic group respecting the taxa included in the analysis (bootstrap 100%).

Comparison of nucleotide diversity values of control region sequences from the ancestral group 2n=42-41 and the derived Rb 2n=38-34 did not show significant differences, even comparing the more polymorphic domains. Evidence so far cumulated indicates that chromosome evolution of *G. griseoflavus* produced a clear genetic differentiation, supporting that 2n=42-41 and 2n=38-34 constitute two (or even more) sibling species. It is noticeable that the chromosomal evolution occurring in *Graomys* does not correlate with the nucleotide diversity of the mitochondrial DNA control region, suggesting that chromosome evolution has occurred in a very short period of time, in agreement with Theiler et al. (1999). These authors, by means of allozymic analysis, proposed that fixation of chromosome fusions could be very fast.

On the base of the average base substitution of cyt *b*, Catzeflis et al. (1992) proposed an approximated time scale of 7-8% substitutions per million years. For this gene, *Graomys* showed an average base substitution of 11%, suggesting that the karyomorphic divergence would have occurred about 1.5 million years ago (Catanesi et al., 2002), time long enough to expect higher control region nucleotide diversity than the observed here. Avise (1986) proposed a theoretical model of stochastic extinction of matriarchal lineages encompassing speciation events. According to this author, there is high probability for sibling species to be polyphyletic in matriarchal ancestry for about 2-4 k generations after speciation (where k is the carrying capacity of each sibling species). Only later, as lineage sorting through random extinction continues, the probability greatly increases for the sibling species to become monophyletic with respect to one another. In agreement with Avise's proposal one may assume that 2n=38-34 chromosome evolution has also involved stochastic extinction of some matriarchal lineages resulting in the establishment of the current consensus mitochondrial haplotype which, in addition to be shared by all Rb karyomorphs, is clearly distinguishable from 2n=42-41. Studies in several taxa have shown that control region sequence constitutes a polymorphic genetic marker useful to adjust phylogenetic relationships among close species. *Graomys* phylogeny based on complete DNA sequence of this region was in concordance with the chromosome differentiation among 2n=42-41 and 2n=38-34 karyomorphs, reinforcing the single origin of the Rb individuals (Catanesi et al., 2002; Zambelli et al., 2003). It is remarkable the wide geographical distribution of the Rb karyomorphs included in this study when compared to the ancestral 2n=42-41 karyomorphs,

which are restricted to the Espinal and Western Chaco area (Theiler and Blanco, 1996 b). According to Theiler et al. (1999) some selective advantage would have allowed fast dispersal of the 2n=38-37 karyomorphs in the Monte region, a zone unexploited by the 2n=42. These advantages probably were acquired by generation of new coadapted gene complexes in the Rb chromosomes (Theiler et al. 1999). This fact may explain the ample distribution exhibited by the 2n=38-34 individuals here analysed.

Chromosomal evolution in rodent taxa have been widely studied (for a review see Slamovits and Rossi, 2002). Different models such as *Microtus* (Modi, 1993), mole rats (Nevo et al., 1994) and house mice (Redi and Capanna, 1988; Nachman and Searle, 1995; Garagna et al., 2001) have been extensively analyzed. In *Graomys* it was proposed that centric fusions appeared in a non-random sequence (Zambelli et al., 1994) becoming this taxa a different model of chromosome evolution compared to that described in *M. domesticus* (Britton-Davidian et al., 1989; Nachman et al., 1994; Riginos and Nachman, 1999).

The common shrew *Sorex araneus* constitutes one of the Rb mammal taxa more deeply studied (Searle and Wójcik, 1998). Findings in cyt *b* gene from Poland populations showed no population subdivision (F_{st} values equal to zero) and lack of phylogeographical structure, proposing a "sudden expansion" model in agreement with the White's (1978) stasipatric model of chromosome evolution (Ratkiewicz et al., 2002). According to Nachman et al. (1994), the lack of concordance between phylogeny and geography is expected if in present-day populations ancestral polymorphisms are still segregating. In *Graomys*, analysis of specimens for cyt *b* (Catanesi et al., 2002) and control region sequences (present study) did not exhibit phylogeographical structure; in fact, trees showed clades including animals from distant localities. Comparative studies between chromosome races of *Sorex araneus* from Sweden showed lack of mitochondrial DNA divergence proposing that most haplotypes arose in situ and that the populations have passed a bottleneck and undergone a rapid size expansion (Andersson et al. 2005). Control region sequences from *Graomys* Rb animals exhibited lack of DNA differentiation (similar nucleotide diversity) and no population subdivision, suggesting that occurrence and fixation of chromosome rearrangements were produced during such a short evolutionary time that did not cumulate as many point mutations as one should expect for this highly evolving molecule, probably indicating the recentness of the chromosomal differentiation (or sudden expansion) of the *Graomys* Rb karyomorphs.

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