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Autosomal rearrangements in *Graomys griseoflavus* (Rodentia): a model of non-random Robertsonian divergence

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The south American rodent *Graomys griseoflavus* exhibits a remarkable chromosome polymorphism as a consequence of four Robertsonian fusions. Focusing on the genetic analysis of the taxon, genome organization of all karyomorphs was studied at chromosome and molecular organization level. Cytogenetic (G, NOR and Re banding) and molecular (satellite and mitochondrial DNAs) events accompanying chromosome divergence allowed tracing a phylogenetic relationship among all karyomorphs. Available data led to propose that chromosome evolution of *G. griseoflavus* occurred in a non-random sequence of centric fusions, supporting the hypothesis of single origin for Robertsonian karyomorphs.

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Robertsonian fusions and reproductive consequences

Robertsonian fusion (RF) is one of the most frequent chromosomal rearrangements in mammals. It implies the joining of two terminal-centromere chromosomes, either acrocentric or telocentric, producing a new biarmed chromosome. Species with Robertsonian polymorphism have been considered as models for chromosome evolution and also for speciation studies (KING 1993).

Graomys griseoflavus (Waterhouse, 1837) is a phyllotine murid rodent widely distributed in Argentina, with a remarkable Robertsonian autosomal polymorphism. In these taxa ZAMBELLI et al. (1994) showed karyomorphs with diploid numbers equal to 42, 41, 38, 37, 36, 35, and 34. In the distribution area 2n = 42–41 individuals inhabit the "Espinal" and "Western Chaco" phytogeographic regions located in central Argentina, while animals of complex 2n = 38, 37, 36, 35 and 34 (2n = 38–34) are found mainly at "Monte" region, in the western-central area of the country. According to THEILER and BLANCO (1996a) and TIRANTI (1998) there are no significant geographic barriers separating different populations of Graomys; in fact, these authors described narrow overlapping zones in some regions. Based on the cytogenetic and geographical distribution features, one could presume that Graomys agrees with the sympatric chromosomal speciation model proposed by WHITE (1978). On the other hand, ZAMBELLI et al. (1994) proposed that the chromosomal variability observed in Graomys is the consequence of four autosomal Robertsonian fusions (RF): RF1-6, RF2-5, RF15-17 and RF16-18 (Table 1). In that report, cytogenetic features of these RFs were summarized as follows: a) both of RF15-17 and RF16-18 (present in 2n = 34–38 complex) were always found in homozygous (Hm) state; b) all of the 2n = 36 and 37 individuals were found Hm and heterozygous (Ht) respectively, for RF1-6; and c) all of the 2n = 34 and 35 animals were Hm for RF1-6, and Hm and Ht respectively, for RF2-5 (ZAMBELLI et al. 1994). These findings led to suggest a sequential occurrence of RFs where HmRF15-17 and HmRF16-18 were first established and then followed by the occurrence of RF1-6; RF2-5 appeared once HmRF1-6 was fixed. Based on this, the authors proposed a sequence of Robertsonian events producing the karyotypic divergence currently observed in Graomys taxa. Accordingly, two lines derived from the 2n = 42 ancestral karyomorph: one in 2n = 41 individuals with very low frequency, and other, in 2n = 38 specimens. The 2n = 38 karyomorph has been the consequence of a reduction in

Table 1. *Robertsonian fusions (RF) found in each karyomorph of G. griseoflavus.*

2n	RF1-6	RF2-5	RF15-17	RF16-18
42	–	–	–	–
41	Ht	–	–	–
38	–	–	Hm	Hm
37	Ht	–	Hm	Hm
36	Hm	–	Hm	Hm
35	Hm	Ht	Hm	Hm
34	Hm	Hm	Hm	Hm

Ht: heterozygous.
Hm: homozygous.
–: absence of the RF.

diploid number occurred in 2n = 42 by two homozygous RFs (RF15-17 and RF16-18). The 2n = 37, 36, 35, and 34 karyomorphs appeared from 2n = 38, by a non-random downward sequence accounted for RF1-6 and RF2-5 (Fig. 1; ZAMBELLI et al. 1994). The suggested ancestry of the 2n = 42 karyomorph was initially based on the proposal of GARDNER and PATTON (1976), who stated that karyotypic evolution in Neotropical rodents decreases the chromosomal number through RFs. The ancestry of 2n = 42 was afterwards reinforced by studies on repetitive DNA sequences (see below).

THEILER and BLANCO (1996a, 1996b) also analysed the reproductive behavior of *G. griseoflavus*, finding interfertility among 2n = 36, 37, and 38 karyotypes through laboratory mating tests. Those studies also showed the existence of reproductive isolation between 2n = 36–38 complex and 2n = 42 karyomorph: crosses between 2n = 36–38 males and 2n = 42 females were unreproductive, while crosses between 2n = 42 males and 2n = 36–38 females produced hybrid descendants. All hybrid males and 80% hybrid females were sterile, while the remaining 20% showed very low fertility in backcrosses (THEILER and BLANCO 1996a).

Under Mendelian inheritance for chromosomal segregation, one should expect all hybrids 2n = 36–38 × 2n = 42 to be heterozygous for both RF15-17 and RF16-18. On the other hand, in order to explain the karyotypic divergence from 2n = 42 to 2n = 38, one may assume the occurrence of intermediate karyomorphs 2n = 39 and 41, heterozygous for RF15-17 or RF16-18. In over 100 animals so far studied, only homozygous individuals for such RFs were found. The results of laboratory crosses (2n = 36–38 × 2n = 42) along with the cytogenetic findings in natural populations strongly suggest that animals heterozygous for RF15-17 and/or HtRF16-18 have some negative feature. There is evidence that heterozygous RFs may be involved in reproductive failure (and in some cases in speciation processes) if produce meiotic non-disjunction (KING 1993). It has been suggested that gametic cell precursors bearing RF15-17 and RF16-18 in heterozygous state fail to segregate during meiosis, affecting fertility in heterozygotes and producing a post-zygotic reproductive barrier (ZAMBELLI et al. 1994). However, other studies have shown that the isolation mechanisms between 2n = 36–38 and 2n = 42 karyomorphs are not restricted to post-zygotic barriers. Sexually receptive females from both groups showed a highly significant preference for olfactory stimuli from conspecific males (THEILER and BLANCO 1996b).

Molecular evolution of highly repetitive DNA sequences

The molecular mechanisms involved in Robertsonian rearrangements have not been yet clarified. However, indirect evidence indicates that highly repetitive DNA or satellite DNA (satDNA) would play a role in the origin of chromosome rearrangements influencing or modulating the frequency of chromosome changes. Probably, the presence of long stretches of satDNA sequences located on non-homologous chromosomes may provide favorable cytological conditions for the occurrence of Robertsonian fusions (BAKER and BICKHAM 1986; REDI et al. 1990; GARAGNA et al. 1993, 2001). In all Graomys karyomorphs, digestion of genomic DNA with the restriction enzyme *EcoR*I released a repeated ladder with a 250 bp main unit, named EG250 (ZAMBELLI and VIDAL-RIOJA 1995). This unit was used as probe for southern-blot analysis of *EcoR*I-digested genomic DNA from all Graomys karyomorphs and from related sigmodon-

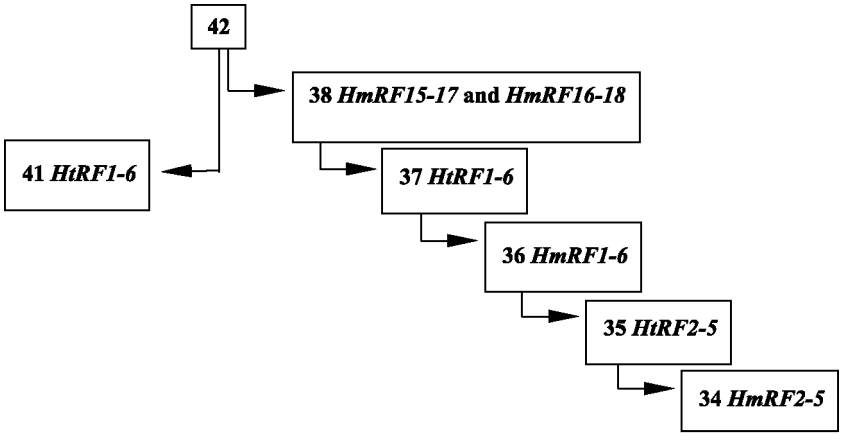

Fig. 1. Sequence of Robertsonian fusions occurred during *G. griseoflavus* chromosome divergence from the ancestral karyomorph 2n = 42.

tine rodents: *Calomys musculinus*, *Phyllotis darwini*, *Eligmodontia typus*, *Oryzomys longicaudatus* and *Akodon mollis*. The interkaryomorphic comparison showed that satEG250 has a homogeneous molecular organization pattern among all Graomys karyomorphs; contrarily, this satellite is absent in all related species (ZAMBELLI and VIDAL-RIOJA 1995). In the divergence tree of Phyllotinii tribe, Graomys is recognized as the most recently appeared genus (GARDNER and PATTON 1976). Therefore, satEG250 might be considered as marker of the divergence of Graomys genus from the Phylotinii tribe. This observation suggests the monophyly of the genus (or at least of the species studied) in concordance with the phylogenetic relationship traced by sequencing of mitochondrial cytochrome b gene (see below).

Methylation pattern of satEG250 was analyzed by DNA cleavage of all Graomys karyomorphs with the isoschizomers *Msp*I and *Hpa*II, and subsequent southern-hybridization with EG250 sequences. In 2n = 42 specimens the autoradiograms corresponding to *Msp*I showed a 250-mer tandem organization with a 1 kb long smallest fragment, while 2n = 38–34 individuals showed two bands of 0.2 and 0.3 kb. *Hpa*II showed no digestion in 2n = 42 animals while a single 0.3 kb band was found in the 2n = 38–34 group. Accordingly, in 2n = 42 individuals the *Msp*I/*Hpa*II sites within satEG250 appeared fully methylated (not digested by *Hpa*II) whereas in the 2n = 38–34 group they were partially demethylated (ZAMBELLI and VIDAL-RIOJA 1999). Further analysis of the molecular organization of satDNA can be performed combining two enzymes. In Graomys this analysis revealed that satEG250 is heterogeneous and comprises at least two subsets of sequences (ZAMBELLI and VIDAL-RIOJA 1995).

In situ hybridization of satEG250 showed that in 2n = 42 karyomorph the repeated family is located at the centromeres of all chromosomes, except for acrocentrics 7 and 14; in 2n = 36 karyomorph positive centromeres were detected in all chromosomes except for pairs 7, 14, and RF16-18 (ZAMBELLI and VIDAL-RIOJA 1995).

Another family of repeated DNA sequences found in *G. griseoflavus* was the satHpa3.2 (ZAMBELLI and VIDAL-RIOJA 1999). DNA digestion from 2n = 42–41 karyomorphs with *Hpa*II showed four faint ethidium bromide stained bands (3.0, 3.2, 3.4, and 3.6 kb), while the 2n = 38–34 karyomorphic group showed one discrete band of 3.2 kb (named Hpa3.2) which was later isolated and used as probe. Genomic DNA from all Graomys karyomorphs and the close related phyllotines *P. darwini* and *E. typus*, were digested with *Hpa*II and southern-hybridized with the Hpa3.2 fragment. Comparisons between 2n = 42–41 and 2n = 38–34 groups revealed interkaryomorphic differences. Thus, 2n = 42–41 individuals exhibited two low-intensity bands of 3.0 and 3.2 kb, while 2n = 38–34 animals showed a high-intensity 3.2 kb band and its less-intense dimer. *E. typus* showed a pattern identical to 2n = 42–41 Graomys, while *P. darwini* showed no hybridization signal. Within the phyllotine tribe, the molecular evolution of the Hpa3.2 sequences may fit the divergence tree proposed by GARDNER and PATTON (1976). According to these authors, Phyllotis and Eligmodontia are ancestral taxa with respect to Graomys, which is the more recently appeared genus. The organization and amplification degree of the Hpa3.2 sequences in *E. typus* and Graomys 2n = 42–41 karyomorphs are similar, suggesting that Hpa3.2 sequences appeared initially in Eligmodontia and 2n = 42 Graomys karyomorph. Afterwards, these repetitive DNA sequences were markedly amplified as a tandem repeat in the 2n = 38–34 group. The Hpa3.2 similarity of Eligmodontia (ancestral genus respect to Graomys) and 2n = 42, reinforces the notion that this latter is the ancestral karyomorph to *Graomys griseoflavus* (ZAMBELLI and VIDAL-RIOJA 1999).

Evolution of the nucleolar organizer region (NOR) patterns

In *G. griseoflavus* NOR regions were studied both by silver staining (Ag-NOR) and in situ hybridization (ZAMBELLI and VIDAL-RIOJA 1996). The first method detects transcriptionally active ribosomal genes, and the second one identifies ribosomal genes irrespective of their functional state. In specimens with 2n = 42 it was shown a number of Ag-NOR sites ranging from 8 to 13 with a marked variation in their chromosome location. The polymorphic variants involved the acrocentric pairs 7, 8, 11, and 15, while 3 and 16 pairs showed an invariable Ag-NOR position. In the 2n = 38–34 complex all karyomorphs showed a single Ag-NOR pattern, comprising five NOR-bearing chromosomes (3, 7, 8, 11, and RF15-17) with a total of 10 Ag-NORs. As mentioned, in this karyomorphic group 15 and 17 pairs are involved in HmRF15-17. Therefore, in this Robertsonian pair the Ag-NOR was detected at the telomeres of the long arms of the 15 moiety (ZAMBELLI and VIDAL-RIOJA 1996).

In situ hybridization demonstrated that 2n = 38–34 animals underwent two NOR deletions (ruling out NOR-inactivation) affecting the paracentromeric NOR at 7 and 16 pairs. According to these results the loss of the 7 pair-NOR should have occurred by concerted evolution through mechanisms of unequal crossing-over, while the loss of NOR 16 should has been caused by a deletion during the RF16-18 estab-

lishment (ZAMBELLI and VIDAL-RIOJA 1996). Homogenization of NOR pattern within all Robertsonian karyomorphs might be another consequence of the chromosome divergence triggered by the occurrence of HmRF15-17 and HmRF16-18.

Phylogenetic relationship among Robertsonian karyomorphs by mitochondrial DNA analysis

Mitochondrial DNA is a rapidly evolving molecule, maternally inherited and not linked to any nuclear marker. Therefore, areas of congruence of phylogenetic trees based on nuclear and mitochondrial DNA markers may reflect past relationships. Variation in the mitochondrial cytochrome b (cyt b) DNA sequence has provided phylogenetic resolution for several orders of mammalian taxa (IRWIN et al. 1991; NACHMAN et al. 1994; SMITH and PATTON 1999).

Recently, CATANESI et al. (2002) carried out a comparative sequencing of cyt b fragments from all Graomys karyomorphs; the parsimony trees obtained depicted two well defined clades: one including 2n = 42–41 animals (100% bootstrap) and the other with 2n = 38–34 individuals (100% bootstrap). When compared to *Eligmodontia typus* and *Phyllotys xanthopygus* they also found that data support the monophyly of all Graomys karyomorphs (98% bootstrap). From cyt b results one question emerges: why 2n = 42 individuals having the current 2n = 38–34 cyt b consensus haplotype were never found. 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 (AVISE 1986). In agreement with AVISE's proposal one may assume that 2n = 38–34 chromosome evolution has also involved the stochastic extinction of some matriarchal lineages resulting in the establishment of the current consensus mitochondrial haplotype. This haplotype, which is shared by all Robertsonian karyomorphs, clearly differs from the 2n = 42–41 consensus haplotype (CATANESI et al. 2002).

Final remarks

Mammalian taxa with Robertsonian polymorphism have been widely studied to explain the mechanism of different chromosome processes such as fusion, evolution, and speciation. Many rodent models have been used around the world including Microtus (MODI 1993), mole rats (NEVO et al. 1994) and house mice (REDI and CAPANNA 1988; NACHMAN and SEARLE 1995).

Molecular-cytogenetic analysis of chromosome evolution of *Graomys griseoflavus* showed a marked differentiation between 2n = 42–41 and 2n = 38–34 karyomorphic groups. Based on allozyme patterns and reproductive behavior THEILER (1996b) and THEILER et al. (1999) revised the taxonomic status of Graomys species and reassigned them to: *Graomys centralis*, for 2n = 42 specimens, and *Graomys griseoflavus*, for those of the 2n = 38–36 complex, although the author did not include in the revision 2n = 41, 35 and 34 individuals. The genetic differentiation observed with nuclear and non-nuclear DNA markers, supports the observation that 2n = 42–41 and 2n = 38–34 constitute two (or even more) sibling species. Despite the data evidencing that 2n = 42 and 38–36 karyomorphs represent different species, TIRANTI (1998) argued that nomenclature condition of *Graomys griseoflavus* has been not yet addressed, particularly for the assignment of the available names *centralis*, *griseoflavus*, *edithae* and *medius*.

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 satDNA organization. Analysis of NOR locations by Ag-NOR and in situ hybridization revealed that 2n = 42 exhibited highly variable NOR patterns both in number and chromosome location, while 2n = 38–34 karyomorphic group exhibited a single NOR pattern. The latter animals, compared to 2n = 42 karyomorphs, have undergone two NOR deletions (ZAMBELLI and VIDAL-RIOJA 1996). On the other hand, Graomys chromosomal divergence has been correlated with the molecular organization of two satDNAs (satEG250 and satHpa3.2). When these sequences were analyzed in all karyomorphs a clear distinction between 2n = 42–41 and 2n = 38–34 was found. The differentiation was observed at the level of methylation pattern of satEG250, and the molecular organization of satHpa3.2 (Fig. 2).

Heterochromatin comprises long arrays of satellite DNA forming clusters of over-condensed chromatin located around centromeres and telomeres (JOHN 1988). In Graomys, investigation of the C-bands produced barely noticeable heterochromatic blocks (ZAMBELLI and VIDAL-RIOJA 1995). Further assays were performed by restriction enzyme banding (Re-banding) (BIANCHI and BIANCHI 1987). Among a set of enzymes, *Mbo*I and *Alu*I detected centromeric heterochromatic Re^+ blocks (ZAMBELLI and VIDAL-RIOJA 1995). In 2n = 42 animals the Re^+ blocks were seen in all of the chromosomes involved in Robertsonian fusions. Moreover, Re-banding in

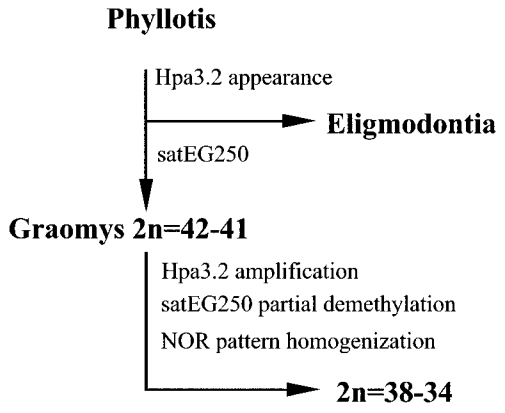

Fig. 2. Genomic events accompanying the divergence of *G. griseoflavus* karyomorphs and the related taxa Phyllotis and Eligmodontia.

2n = 36 karyomorphs met the 2n = 42 pattern, except for one RF1-6 homologue and both HmRF15-17 and HmRF16-18, which showed no blocks (ZAMBELLI and VIDAL-RIOJA 1995). Thus, Re^+ heterochromatin localization partially coincided with the satEG250. Accordingly, three groups of chromosomes were defined: a) positive EG250 and Re-banding; b) positive EG250 and negative Re-banding; and c) negative EG250 and Re-banding. In 2n = 36 karyomorph of Graomys, one RF1-6 homologue and HmRF15-17 represent the group b) of chromosomes. Therefore, satEG250 was suggested to be present forming two different sets: i) with heterochromatic conformation; and ii) without heterochromatic conformation (ZAMBELLI and VIDAL-RIOJA 1995).

The house mouse *Mus domesticus* constitutes one of the most largely studied mammal model for Robertsonian (Rb) chromosome differentiation. Several hypotheses (even opposite) have been proposed in order to explain the evolutionary origin of Mus Rb races. One of these, suggests that Rb populations arose in restricted areas from which they spread to the present locations (WINKING et al. 1988; BAUCHAU 1990; NACHMAN et al. 1994; RIGINOS and NACHMAN 1999). This proposal suggests that all Rb populations may be more closely related to each other than to a standard karyotype population. Accordingly, the mechanism producing Rb rearrangements should be present in a single *M. domesticus* lineage. In Graomys, phylogenetic trees from cyt b sequences showed Rb karyomorphs of the 2n = 38–34 complex grouped in a single clade, while the ancestral 2n = 42 and 2n = 41 karyomorphs formed a different one (CATANESI et al. 2002). This is consistent with the hypothesis of single origin for Rb karyomorphs. With the aim to investigate further the phylogeny of Graomys karyomorphs, we sequenced the complete mitochondrial D-loop region (unpubl.). Basically, analysis of the data showed trees with two main clusters, one including the 2n = 42–41 karyomorphs and the other with the 2n = 38–34. This topology is similar to the obtained by cyt b sequencing, with both clades having very high bootstrap values. These findings reinforce the proposal for the single origin for Rb animals. In spite D-loop region is a highly evolving sequence, phylogenetic trees did not exhibited a clear differentiation among 2n = 38–34 animals, indicating that Rb animals could constitute a genetically homogeneous group (unpublished). THEILER et al. (1999) proposed that fixation of RF15-17, 16-18, and 1-6 in the 2n = 38–36 group has occurred without a bottleneck effect. Opposed to THEILER's proposal, the concordance between nuclear and mitochondrial DNA data supports the single origin hypothesis for Graomys Rb races; therefore, the occurrence of a founder effect during chromosomal differentiation cannot be rule out. Although nuclear and mitochondrial genomes have quite different evolutionary dynamics, cyt b differentiation between 2n = 42–41 and 38–34 karyomorphic groups does agree with the interkaryomorphic differences described for NOR patterns and repetitive DNA sequences organization.

Based on the cytogenetic analysis of *Graomys griseoflavus* a non-random sequence of Robertsonian rearrangements has been set up where the 2n = 38–34 complex arises from the ancestral 2n = 42 karyomorphs (Fig. 1). In other words, it seems that in Graomys the occurrence of a given RF needs to be preceded by a different one. The key chromosomal event during Graomys divergence was the establishment of HmRF15-17 and HmRF16-18, which are the common feature in the 2n = 38–34 complex. Since these RFs are only fixed in homozygous state (heterozygotes may have reproductive failure) it is possible that they could provide the suitable genetic environment for the occurrence of RF1-6 and RF2-5. In the same way, RF2-5 has been so far always found together with HmRF1-6. The only exception to these observations is the very lowly frequent 2n = 41 karyomorph, carrying HtRF1-6 with absence of both RF15-17 and RF16-18. However, satHpa3.2 and cyt b sequencing analysis, showed that 2n = 41 is closely related to 2n = 42, forming a divergent line clearly differentiated from the Robertsonian 2n = 38–34 complex.

Available evidence indicates that *G. griseoflavus* is undergoing a chromosome evolution process influencing on its speciation. This Robertsonian group, as others do, constitutes a useful model for chromosome evolution studies. Although Graomys has lower frequency of chromosome translocations than house

mouse, a distinctive characteristic of this taxon is the occurrence of Robertsonian fusions in a non-random sequence. For the house mouse, the very high frequency of Rb chromosomes, and the random involvement of the telocentrics in the centromeric fusion could be produced by "inherent genomic traits" such as the clustering of heterochromatic regions, among other factors (REDI et al. 1990; GARAGNA et al. 2001). For Graomys the major satEG250 was found both in heterochromatic and non-heterochromatic conformation, being the first form present in the chromosomes able to fuse. These heterochromatic blocks would be composed by a particular subset of satEG250, which probably constitutes the molecular key to the occurrence of non-random Robertsonian fusions.

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