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Molecular events during chromosomal divergence of the South American rodent *Graomys griseoflavus*

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Graomys griseoflavus (Waterhouse, 1837) is a South American phyllotine rodent having an autosomal polymorphism produced by a sequence of Robertsonian fusions. The chromosomal divergence was correlated with the molecular organization of two satellite DNA families (EG250 and Hpa3.2) and the second internal transcribed spacer (ITSII) of ribosomal DNA. These studies were performed by Southern hybridization on all *G. griseoflavus* karyomorphs and other closely related cricetids. When *Graomys* karyomorphs were compared, interkaryomorphic differences were found at the methylation pattern level of EG250 satellite and the molecular organization of Hpa3.2 satellite and ITSII. The findings support the hypothesis that *Graomys griseoflavus* karyomorphs are evolving separately, probably driving a chromosomal speciation process between 2 n = 42–41 and 2n = 38–34 individuals.

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Introduction

Graomys griseoflavus (Waterhouse, 1837) is a phyllotine rodent widely distributed in Argentina which shows remarkable autosomal polymorphisms modifying both diploid (2n) and fundamental number of chromosomes (Zambelli *et al.* 1994). Thus far, these authors described 2n = 42, 41, 38, 37, 36, 35 and 34 karyomorphs and proposed a chromosomal divergence pathway accounted by four sequential Robertsonian fusions (RF): RF1–6, RF2–5, RF15–17 and RF16–18. Cytogenetic and breeding data suggest that 2n = 42 is the ancestral karyomorph from which two karyomorphic lines derived: one producing very low frequency 2n = 41 individuals and the other 2n = 38 specimens (Zambelli *et al.* 1994). From the latter, and in a non-random downward sequence, the 2n = 37–34 karyomorphs have appeared (Zambelli *et al.* 1994).

In the area studied, the 2n = 42 individuals inhabit the phytogeographic region called "espinal" located in central Argentina and the complex 2n = 38–34 mainly occupies the crescent shaped area called "monte", west and south of the "espinal". There are no significant geographic barriers separating different populations of *Graomys*, in fact, narrow overlapping zones occur in some regions (Theiler and Blanco 1996, Tiranti 1998).

In the laboratory, matings between 2n = 42/41, 38/37, 38/36 and 37/37 resulted in F1 and F2 fertile progenies while matings between 2n = 42/38–36 individuals failed to breed or gave sterile hybrids (Zambelli *et al.* 1994, Theiler and Blanco 1996).

Due to their high evolutionary rate, analysis of the molecular organization of highly and middle repetitive DNA sequences have proved to be useful to construct divergence pathways in several taxa. It has been fairly well demonstrated that these sequences evolve in concert, determining intraspecific sequence homogeneity (Dover 1982, Arnheim 1983). However, if in some specimens of a given population, individual members of the repetitive sequences evolve independently, the DNA family becomes heterogeneous within the taxon, indicating in most of the cases a taxonomic divergence process. Accordingly, the results of satellite DNA and ribosomal DNA spacers analysis have been used to trace relationships among recently diverged taxa (Hardies 1986, Suzuki *et al.* 1987, 1994, Hamilton *et al.* 1990, Allard and Honeycutt 1991, Hillis and Dixon 1991, Graur 1993, Modi 1993, Kass *et al.* 1996).

In this paper, we analysed comparatively in all *Graomys griseoflavus* karyomorphs the genomic organization of two repetitive DNA families (EG250 and Hpa3.2) and the second internal transcribed spacer (ITSII) of the ribosomal DNA unit. The molecular findings were correlated with our previously proposed chromosomal divergence pathway, reinforcing the hypothesis that *Graomys griseoflavus* karyomorphs are evolving separately, probably driving a chromosomal speciation process between 2n = 42–41 and 2n = 38–34 groups.

Material and methods

Specimens examined

Graomys griseoflavus karyomorphs were collected in the following locations of Central Argentina: Santiago Temple, Laguna Larga and Deán Funes in Córdoba Province (10 individuals with 2n = 42 and 1 with 2n = 41); Salicas in La Rioja Province and La Carrera in Catamarca Province, approximately 600 km northwest from Santiago Temple and Laguna Larga and approximately 100 km west from Deán Funes (7 individuals with 2n = 38, 4 with 2n = 37 and 2 with 2n = 36); and Divisadero Largo in Mendoza Province, approximately 450 km south from Salicas and 600 km west from Santiago Temple and Laguna Larga (2 individuals with 2n = 36, 2 with 2n = 35 and 1 with 2n = 34). All individuals were karyotyped as previously described (Zambelli *et al.* 1994).

DNA analysis

DNA was extracted from liver tissues as previously described by Zambelli and Vidal-Rioja (1995). DNA from all karyomorphs was digested with restriction enzymes (Gibco-BRL) following manufacturers instructions. DNA fragments were separated in 1% agarose gels and processed for Southern blotting and hybridization according to Sambrook *et al.* (1989). The DNA fragment probes EG250 and Hpa3.2 were isolated by agarose gel purification digesting *Graomys* genomic DNA with EcoRI and HpaII, respectively. For ribosomal DNA unit mapping, human recombinant plasmids pA1 (Erickson *et al.* 1981) and pR18S (Manuelidis and Ward 1984) including 28S and 18S rDNA genes, respectively were used as probes. For DNA ribosomal mapping drawing, human pattern was used as reference. All DNA probes were labeled with α-^{32}P-dCTP (Du Pont-NEN) by the nick translation procedure. Hybridizations were performed in a mixture of 1.5 × SSPE (1 × SSPE: 150mM NaCl, 10mM $NaH_2PO_4.H_2O$, 1mM EDTA pH=7.4), 1% SDS, 0.5% non-fat dry milk and 100μg/ml of sonicated salmon sperm DNA. Radiolabeled probes were hybridized with the DNA immobilised on nylon

membranes (Z Probe, Bio Rad) at 65°C overnight. Afterwards, filters were washed twice for 20 min with 0.5 × SSC/0.5% SDS at room temperature and once at 60°C.

Results

Organization of the repetitive DNA families EG250 and Hpa3.2

EG250 family

EcoRI digested DNAs from all *Graomys* karyomorphs share an identical 250-mer ladder with a main unit of 250 bp named EG250 (Zambelli and Vidal-Rioja 1995). Southern comparisons of this repetitive sequence indicated that its molecular organization is homogeneous within *Graomys* karyomorphs and species- specific of this taxon. When compared with closely related phyllotines such as *Phyllotis darwini* (2n = 38) and *Eligmodontia typus* (2n = 44), EG250 sequences were not found (Zambelli and Vidal-Rioja 1995). Analysis of methylation pattern of EG250 sequences was performed digesting DNA with the isoschizomers MspI and HpaII restriction enzymes. The former acts either if the internal cytosine of the recognition sequence is methylated or demethylated, while the second does it only if the cytosine is demethylated. The MspI and HpaII digests from all *Graomys* karyomorphs were blotted and Southern hybridized with EG250 labeled probe isolated from both 2n = 42 and 2n = 36 karyomorphs. In 2n = 42 specimens, the MspI autoradiograms showed a 250-mer tandem organization with the smallest fragment being 1 kb long, while the 2n = 38–34 individuals showed two bands of 0.2 and 0.3 kb (Fig. 1). The HpaII digests showed no bands in 2n = 42 animals while a single 0.3 kb band was characteristic of the 2n = 38–34 group. The results described were identical when using either 2n = 42 or 2n = 36 EG250 isolated probes.

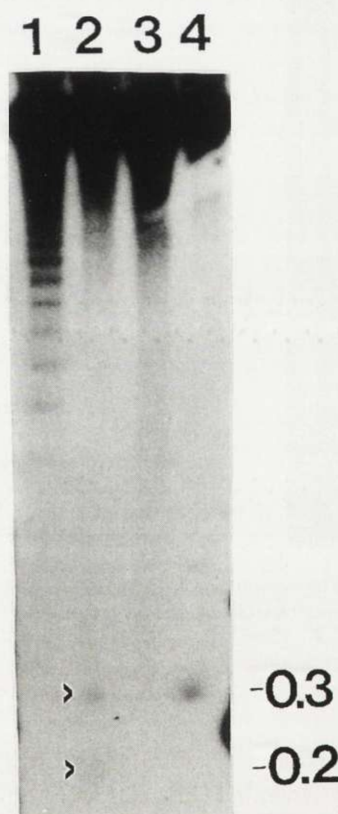

Fig. 1. Southern blot hybridization with EG250 radiolabeled probe on DNA digested with the isoschizomers MspI (1,2) and HpaII (3,4). Lanes 1 and 3 correspond to DNA from 2n=42 karyomorph and lanes 2 and 4 of 2n=34 karyomorph. Notice the MspI type B pattern (1) and the absence of HpaII digestion (3) in 2n = 42 karyomorph. The discrete fragments detected with each enzyme are pointed.

Hpa3.2 family

Digestion of 2n = 42–41 *Graomys* DNA with the restriction enzyme HpaII showed faint ethidium bromide stained bands of 3.0, 3.2, 3.4 and 3.6 kb, while the 2 n = 38–34 karyomorphic group showed one discrete band of 3.2 kb (named Hpa3.2) which was excised and used as probe (Fig. 2).

Genomic DNA HpaII digests from all *Graomys* karyomorphs and the close related phyllotines *P. darwini* and *E. typus* were Southern hybridized with the

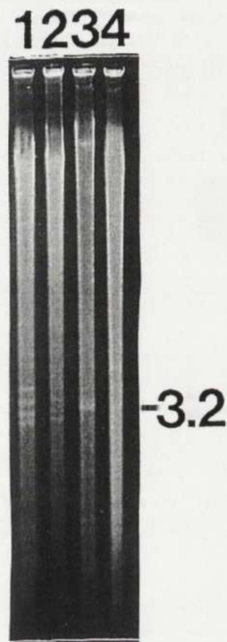

Fig. 2. Fluorescent pattern of HpaII digestions from four *Graomys* karyomorphs: 1 – 2 n = 42, 2 – 2n = 41, 3 – 2n = 38, and 4 – 2 n = 34.

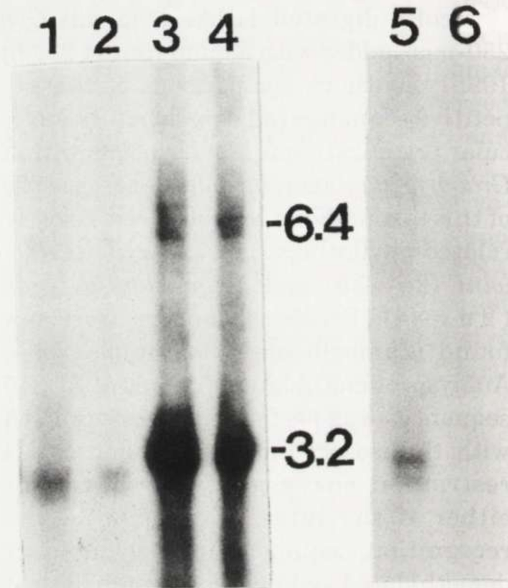

Fig. 3. Southern blot hybridization with Hpa3.2 radiolabeled probe on DNA HpaII digestions from *Graomys*: 1 – 2n = 42, 2 – 2n = 41, 3 – 2n = 38, 4 – 2 n = 34, 5 – *Eligmodontia typus*, and 6 – *Phyllotis darwini*.

labeled Hpa3.2 fragment (Fig. 3). Comparisons between 2n = 42–41 and 2n = 38–34 groups revealed interkaryomorphic differences; thus, 2n = 42–41 individuals presented two low-intensity 3.0 and 3.2 kb bands while 2n = 38–34 animals showed a high-intensity 3.2 kb band and its less-intense dimer (Fig. 3). Regarding the other phyllotine taxa analysed, *E. typus* showed a pattern identical to 2n = 42–41 *Graomys*, while *P. darwini* showed no hybridization signal (Fig. 3).

Restriction map of ribosomal DNA

The ribosomal DNA unit was mapped with the enzymes EcoRI and BamHI, both by single and double digestion, and Southern analysed using either labeled pA1 and

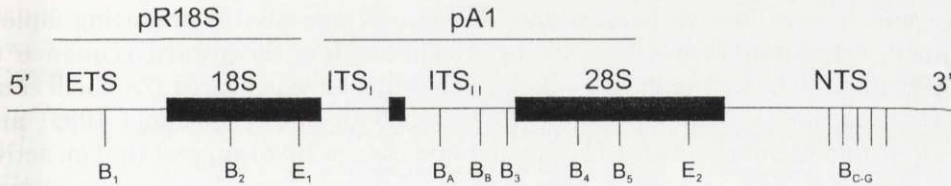

Fig. 4. Restriction map of *Graomys* rDNA unit. E and B indicate restriction sites for EcoRI and BamHI, respectively. Conserved sites are denoted by subscript numbers. Mutated sites are denoted by subscript letters. pR18S and pA1 indicate the regions homologous to the probes.

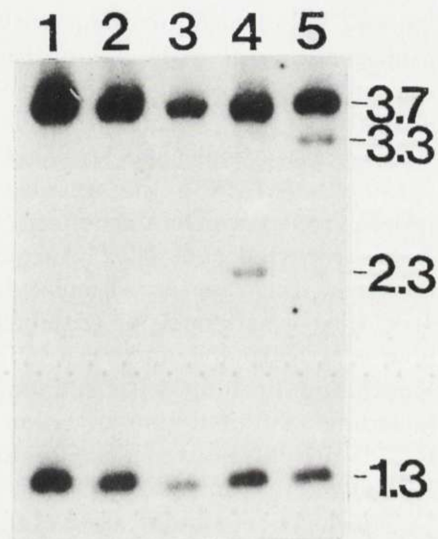

Fig. 5. Southern blot hybridization with pR18S radio-labeled probe on BamHI digestions from *Graomys* karyomorphs: 1 - 2n = 42, 2 - 2n = 41, 3 - 2n = 38, 4 - 2n = 37, and 5 - 2n = 34. Notice the polymorphic bands of 2.3 kb (B_2B_A) detected in all 2n = 37 individuals and 3.3 kb (B_2B_B) observed in 2 n = 36, 35 and 34 karyomorphs.

pR18S human recombinant clones as probes. The restriction map of the regions homologous to the probes are shown in Fig. 4. Differences involving the second internal transcribed spacer (ITSII) and the non-transcribed spacer (NTS) were detected among karyomorphs, the latter by the variable position of one BamHI site (B_{C-G} sites, Fig. 4). Regarding ITSII, the BamHI digestion produced a distinctive faint fragment: B_2–B_A of 2.3 kb found in all 2n = 37 animals analysed and B_2–B_B of 3.3 kb present in all 2n = 36–35–34 individuals studied (Figs 4 and 5).

Discussion

It is widely accepted that Neotropical cricetids evolve by decreasing their diploid numbers via Robertsonian fusions (Gardner and Patton 1976). On this basis we proposed a chromosomal divergence pathway for *Graomys* karyomorphs, considering 2n = 42 as the ancestral karyomorph (Zambelli *et al.* 1994). The 2n = 42/38

divergence occurs by two homozygous RFs (15–17 and 16–18) producing diploid number reduction. From 2n = 38, by a non-random downward sequence of Robertsonian fusions, the 2n = 37–34 karyomorphs have appeared (Zambelli *et al.* 1994). Cytogenetic (Zambelli *et al.* 1994, Zambelli and Vidal-Rioja 1996) and breeding data (Zambelli *et al.* 1994, Theiler and Blanco 1996) suggest that an active karyotype evolution process is occurring in *Graomys griseoflavus*, determining reproductive isolation between 2n = 42–41 and 38–34 groups. The 2n = 42/38–36 matings failed or gave sterile hybrids heterozygous for RF15–17 and 16–18 (Zambelli *et al.* 1994, Theiler and Blanco 1996). Moreover, individuals heterozygous for these RFs have not been found in nature, even in those regions where the 2n = 42 karyomorph overlaps with 2n = 38–34 group. On these grounds, it was proposed that the RF15–17 and 16–18 in heterozygous state would be relevant for the reproductive isolation of 2n = 42–41 and 2n = 38–34 karyomorphs (Zambelli *et al.* 1994).

To assess at molecular level our previous chromosomal evolutionary hypothesis (Zambelli *et al.* 1994) the organization of highly (EG250 and Hpa3.2) and middle (rDNA) repetitive DNA sequences of all *Graomys* karyomorphs were compared.

We reported that EG250 sequences are specific for *Graomys griseoflavus*; moreover, their conserved molecular organization has been demonstrated among all *Graomys* karyomorphs (Zambelli and Vidal-Rioja 1995). In the present paper, however, restriction of this repetitive DNA family with the isoschizomers MspI and HpaII and probing with radiolabeled EG250 sequences showed differences, including a differential methylation pattern. Accordingly, in the 2n = 42 individuals the MspI/HpaII sites of EG250 family appeared fully methylated (no digestion by HpaII) whereas in the 2n = 38–34 group they were partially demethylated.

The degree of amplification of the Hpa3.2 sequences may also be considered as a marker for the karyomorphic groups 2n = 42–41 and 2n = 38–34, with the latter group showing a higher amplification. 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*, the most recently appeared genus. The organization and amplification of the Hpa3.2 DNA family in *E. typus* and *Graomys* 2n = 42–41 karyomorphs are similar, while in *P. darwini* this repetitive DNA family is absent. The evidence suggests that Hpa3.2 sequences appeared initially in *Eligmodontia* with a repetition degree similar to that showed by 2n = 42 *Graomys* karyomorph. Afterwards, in the 2n = 38–34 group these repetitive DNA sequences were markedly amplified as a tandem repeat. The Hpa3.2 similarity of *Eligmodontia* (ancestral genus with respect to *Graomys*) and the 2n = 42 supports our suggestion that the latter is the ancestral karyomorph of *Graomys griseoflavus*.

The partial EG250 demethylation and the differential amplification of Hpa3.2 sequences that occurred in the 2n = 38–34 group indicate that these molecular events took place after the chromosomal divergence 2n = 42/38. Consequently, the presence of homozygous RF15–17 and RF16–18 can be correlated with the genomic

changes observed in the 2n = 38 karyomorph. Moreover, this karyomorph and its downward derivatives 2n = 37–34 share the organization pattern of Hpa3.2 and EG250 sequences which are homogeneous within the 2n = 38–34 group. These results agree with our previous observations on the differential patterns of nucleolar organizer regions (NORs) which in the 2n = 42 individuals the NOR pattern is highly variable while in 2n = 38–34 group it is homogeneous (Zambelli and Vidal-Rioja 1996).

To trace further molecular relationships among the *Graomys* karyomorphs we cautiously analysed the rDNA unit restriction map. These sequences evolve in concert, that is, at a different rate with respect to the rest of the genome and at a different rate if transcribed or non-transcribed regions are considered (Arnheim 1983); for instance, the ITSs evolve at lower rate than NTS. Thus, a putative change affecting the ITS might reflect a divergence instead of a change by chance. Accordingly, we based our conclusions only on the ITSII variations observed. The EcoRI/BamHI restriction mapping showed a variability affecting the ITSII of all 2 n = 37–34 animals analysed. Besides the conserved fragments B_2B_3 (3.7kb) and B_1B_2 (1.3kb), we observed fragments B_2B_A (2.3kb) in all 2n = 37 animals analysed and B_2B_B (3.3kb) in the 2n = 36–34 group. As B_2B_3 and B_1B_2 conserved fragments showed a higher intensity than the polymorphic B_2B_A and B_2B_B, we assumed that the new sites involve only a few copies of the rDNA unit. This assumption may explain the simultaneous presence of conserved and polymorphic fragments.

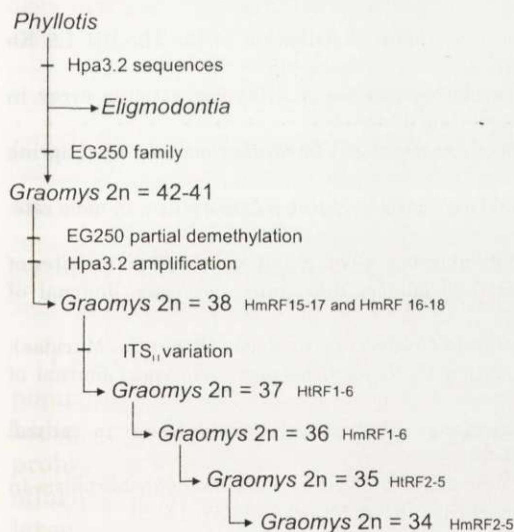

Fig. 6. Molecular events accompanying the phyllotine differentiation and the chromosomal divergence of *Graomys* karyomorphs. The Robertsonian fusions (RF) present in each *Graomys* karyomorph are indicated.

The overlapping of molecular inter-karyomorphic differences observed with the our previously proposed chromosomal evolutionary hypothesis (Zambelli *et al.* 1994) show that the occurrence of Robertsonian fusions are accompanied by demonstrable molecular events (Fig. 6). The systematic status of *Graomys griseoflavus* is still not clear (Tiranti 1998) for which further nomenclatorial analysis should be addressed analysing all the information available so far. The presented molecular data set agree with the previous cytogenetic and breeding data reinforcing the idea that a clear separate karyotype evolution is occurring between 2n = 42–41 and 2n = 38–34 *Graomys griseoflavus* karyomorphs, probably driving a chromosomal speciation process between these karyomorphic groups.

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