

Biological relevance of polyploidy: ecology to genomics

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Whole-genome duplications in South American desert rodents (Octodontidae)

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The discovery of tetraploidy in the red viscacha rat, *Tympanoctomys barrerae* ($4n = 102$) has emphasized the evolutionary role of genome duplication in mammals. The tetraploid status of this species is corroborated here by *in situ* PCR and Southern blot analysis of a single-copy gene. The species meiotic configuration strongly suggests a hybrid derivation. To investigate the origin of *T. barrerae* further, the recently described *Pipanacoctomys aureus* was studied. This 92-chromosome species also has a duplicated genome size, redundant gene copy number and diploid-like meiotic pairing, consistent with an event of allotetraploidization. Phylogenetic analysis of mitochondrial sequences indicates sister-group relationships between these two tetraploid rodents. The new karyotypic data and the phylogenetic relationships suggest the participation of the ancestral lineages of *Octomys mimax* in the genesis of *P. aureus*. The high overall DNA similarity and shared band homology revealed by genomic Southern hybridization as well as matching chromosome numbers between *O. mimax* and the descendant tetraploid species support the notion of introgressive hybridization between these taxa. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 82, 443–451.

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INTRODUCTION

The evolutionary role of genome duplications has motivated intense studies because duplicated gene pathways provide new opportunities for increased body-plan complexity, organismal differentiation and adaptation by recruitment of new genes to new roles (Holland & García-Fernández, 1996; Holland & Chen, 2001; Furlong & Holland, 2004 – this issue). In fact, comparative genomic data are consistent with two rounds of genome duplications (the 2R hypothesis) in vertebrate evolution (Ohno, Wolf & Atkin, 1968; Gu,

Wang & Gu, 2002; McLysaght, Hokamp & Wolfe, 2002; Hokamp, McLysaght & Wolfe, 2003).

The most efficient and sudden way to achieve genome doubling in eukaryotes is through interspecific hybridization and subsequent genome duplication (Otto & Whitton, 2000). Although polyploidy plays a significant role in the hybrid speciation and polyphyletic origin of many angiosperm taxa (Adams & Wendel, 2004; Doyle *et al.*, 2004 – both this issue), most examples in vertebrates involve species that either demonstrate a parthenogenetic mode of reproduction, lack heteromorphic sex chromosomes or have an environmentally induced sex-determining system (Bogart, 1980; Orr, 1990; but Mable, 2004 – this issue). Consequently, whole-genome duplications in extant mam-

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mals have been considered evolutionary irrelevant to speciation and adaptation due to varied disruptions of the dosage compensation mechanism (Goto & Monk, 1998; Otto & Whitton, 2000).

However, in mammals, tetraploidy has been reported also in the desert dwelling rodent *Tympanoctomys barrerae* (Gallardo *et al.*, 1999). This species has the largest chromosome number in a mammal ($4n = 102$) and belongs to a monotypic clade of South American rodents that includes *Octomys mimax* (Honeycutt, Rowe & Gallardo, 2003).

T. barrerae's genome size (16.8 pg DNA) is twice that of most octodontids (*O. mimax* = 8.0 pg, *Octodontomys gliroides* = 8.2 pg, *Aconaemys fuscus* = 7.5 pg) and it has increased cell size associated with genome doubling (Gallardo *et al.*, 2003). This polyploidy event was relatively recent and estimated to have occurred 6.5 Mya (Gallardo & Kirsch, 2001). Nevertheless, the derivation of the totally biarmed karyotype of *T. barrerae* is elusive. Here, we present new cytological and molecular analyses that indicate *T. barrerae*'s tetraploid condition. We also show genome size estimates and additional data which indicate that the recently described *Pipanaoctomys aureus* (Mares *et al.*, 2000) is also a closely related tetraploid species.

MATERIAL AND METHODS

ESTIMATION OF GENE COPY NUMBER

In situ PCR using the highly conserved, single-copy, androgen receptor gene (*AR*) was used to test gene duplication in *T. barrerae* assuming that discrete 'marks' from each chromosome indicate the copy number of a candidate locus (Martínez *et al.*, 1995). By being sex-linked in humans, rodents and monotremes (<http://www.informatics.jax.org/>), this gene (also known as *Tfm*) allowed us to address simultaneously the questions of gene copy number and X-chromosome number. Formalin-fixed interphase nuclei were obtained from the bone marrow of one male and one female *T. barrerae* (Martínez *et al.*, 1995). In addition, interphase nuclei from one male and one female *Mus musculus* were used as controls. Nuclei were fixed in 10% formaldehyde, embedded in paraffin and cut (7 µm) before being mounted on slides. One hundred nuclei were checked at random and mark number was counted on each. The statistical significance of intracellular signal dispersion ($P < 0.05$) was assessed by the χ^2 test conducted separately on 100 nuclei of male and female *T. barrerae* and the control species. Air-dried nuclear spreads on silan-covered slides were pretreated with lysis buffer (100 mM Tris/HCl pH 7.2, 50 mM EDTA, 0.5% SDS). Slides were incubated with pepsin (2 mg mL⁻¹, Sigma-Aldrich) in a humid cham-

ber at 37°C for 45 min. Primers selecting a 145-bp AR fragment (Ty-AR145) were chosen from consensus regions with Clustal X (sense: 5'-AGAGAGCTGCAT CAGTTCAC-3'; antisense: 5'-GTGTGTGGAAATAGA TGGGC-3'). The amplified product using *T. barrerae*'s genomic DNA was cloned into pGEM-T-Easy-vector (Invitrogen), sequenced and aligned to previously published sequences to confirm that only the target gene was amplified (accession number AY249754). *In situ* PCR conditions included 15 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min (Sambrook, Frisch & Maniatis, 1989). The 11-dUTP digoxigenin-labelled product was visualized with phosphatase alkaline-conjugated anti-digoxigenin (Mukai & Appels, 1996). Southern blot analysis was used to corroborate gene multiplicity. The rationale behind it assumes that the lack of restriction sites in the probe region for the enzymes used corresponds to a single locus and that the number of bands on the autoradiograph indicates gene copy number (Small & Wendel, 2000; Adams & Wendel, 2004). Southern blotting used genomic DNA of a male and a female *T. barrerae* that was cut to completion with *Bam*HI. Twelve micrograms of DNA was separated on a 1% agarose gel and transferred to a nitrocellulose membrane (Bio-Rad). Blots were probed with the radiolabelled fragment Ty-AR145 and washed under stringent conditions (Southern, 1975). One female specimen each of *O. mimax*, *O. gliroides* and *P. aureus* were used as controls.

MEIOSIS AND CYTOGENETIC ANALYSES

To investigate chromosome pairing and segregation, conventional meiotic analysis for light microscopy was carried out on 25 meiotic spreads of one adult male *T. barrerae* from El Nihuil (Mendoza Province, Argentina) and four males from the Añelo salt flat (Neuquén Province, Argentina), following Evans, Breckon & Ford (1964). Meiotic analysis of ten meiotic spreads was conducted also on two male *P. aureus* from the Pipanaco salt flat (Catamarca Province, Argentina).

The karyotype of *P. aureus* has not been reported previously. Bone marrow chromosomes of one male and one female *P. aureus* from the Pipanaco salt flat were obtained by the colchicine-hypotonic technique conducted in the field (Verma & Babu, 1995). Ten mitotic plates from each individual were selected for subsequent analyses. Chromosomes were arranged in quadruplets by decreasing size: metacentrics-submetacentrics first, subtelocentrics last. Some chromosomes could only be arranged in pairs. Mitotic and meiotic plates were digitally captured and contrast-enhanced with Photoshop 7.0.

GENOME SIZE ESTIMATES

Gametic estimates of genome size of four male *P. aureus* were obtained from 150 sperm fixed in 3 : 1 methanol–glacial acetic acid. Somatic estimates were assessed in 100 ALFAC-fixed kidney cells (ethanol 85%, formaldehyde 10%, glacial acetic acid 5%) of three specimens. Smears were stained using the fluorescent Feulgen reaction of Itikawa & Ogura (1954). DNA estimates were obtained with a Zeiss MPM 400 scanning microdensitometer set to read the emission signal at 542 nm. Sperm samples of *Mus musculus* and *T. barrerae*, used as internal standards, were stained and run simultaneously with the samples being tested. Statistical analysis was carried out with PHOTAN, provided by Zeiss. DNA content was estimated as reported previously (Gallardo *et al.*, 2003).

Sperm dimensions and genome size are causally connected in mammals. The abnormally enlarged sperm heads of diploid gametes reflect this relationship in rabbits (Beatty & Fehheimer, 1972), bovines (Ferrari *et al.*, 1998) and in *T. barrerae* (Gallardo *et al.*, 2002, 2003). To study this effect in *P. aureus*, semen of four males was extruded from the epididymis and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1% cacodylate buffer (Rodríguez, 1969). Post-fixation was carried out with 1% osmium tetroxide in 0.175 M cacodylate buffer. After dehydration and critical point drying, spermatozoa were coated with gold and examined in a Hitachi H 700 scanning electron microscope. The total length of the head, head width and height were recorded by light microscopy of 100 Giemsa-stained spermatozoa.

PHYLOGENETIC RECONSTRUCTION

The phylogenetic relationships of the octodontids were explored with a 936-bp fragment of the 12S rDNA gene. *P. aureus* DNA sequences (GenBank accession number AY249753) were added to previous data (Honeycutt, Rowe & Gallardo, 2003). *Abrocoma cinerea* (Abrocomidae) was used to root the tree. All phylogenetic analyses were conducted using PAUP* 4.0b (Swofford, 1999). Neighbour-joining trees were built using the HKY85 model (Hasegawa, Kishino & Saitou, 1991). Maximum parsimony (MP) analyses were conducted using the heuristic search option. Support for individual nodes was assessed with 1000 bootstrap replications (Felsenstein, 1985). Maximum-likelihood (ML) analyses were also conducted and the most appropriate substitution model for the data set was determined with Modeltest v.3.04 (Posada & Crandall, 1998). Bootstrap support for the ML tree was determined using 100 fast-addition replications.

INTERSPECIFIC GENOMIC HYBRIDIZATION

Introgressive hybridization was investigated by genomic Southern hybridization as exemplified in birch (Thórsson, Salmela & Anamthawat-Jónsson, 2001). For such a purpose, total genomic DNA from liver samples of *O. mimax*, *O. gliroides*, *T. barrerae* and *P. aureus* was prepared according to standard procedures (Sambrook *et al.*, 1989). The successive outgroups for the Octodontidae (Ctenomyidae and Abrocomidae), and the distantly related *Mus musculus*, were also included in the analyses. The total genomic DNA of *P. aureus* was used as a probe to block competitively (and hybridize to) the total genomic DNA of the target species to test for high levels of overall DNA homology. Complete digestion of genomic DNA was carried out with a ten-fold excess of restriction enzyme and overnight incubation at 37°C. One microgram of genomic DNA was fractionated in a 1% agarose gel, stained with ethidium bromide, electrotransferred onto a nitrocellulose membrane, denatured and fixed by baking at 80°C for 90 min. After prehybridization and hybridization to ³²P-labelled total genomic DNA, filters were exposed to autoradiographic film. To test for shared homology, the total genomic DNA of *P. aureus* (the labelled probe) was hybridized to a Southern blot of *Dra*I-digested genomic DNA of different octodontid species (the targets), following the protocols of Pašakinskiene *et al.* (1998) and Thórsson *et al.* (2001). At the same time, unlabelled genomic DNA of *O. mimax* was applied to block common sequences in the target species. Reverse experiments, using the same DNAs and experimental conditions, but probing with genomic DNA of *O. mimax* and blocking with genomic DNA of *P. aureus*, were also conducted (Thórsson *et al.*, 2001). Equal amounts of DNA digests (1 µg DNA) were loaded to the gels.

RESULTS

GENE COPY NUMBER

Four marks (*in situ* PCR signals) were detected in 14% of female nuclei of *T. barrerae*, 17% had three marks, 23% had two marks and 46% had only one mark. By contrast, one and two marks were observed in 55% and 45% of female nuclei of *Mus musculus*, respectively. Three marks were detected in 22% of male nuclei of *T. barrerae*, 30% had two marks and 48% had only one mark. Only one mark was observed in 96% of the control cells. The statistical difference of mark number distribution between *M. musculus* and *T. barrerae* was highly significant (males: $\chi^2_{(2)} = 452$, $P < 0.001$; females: $\chi^2_{(3)} = 287$, $P < 0.001$), indicating a biological difference associated with locus number in these species. Gene duplication was corroborated by

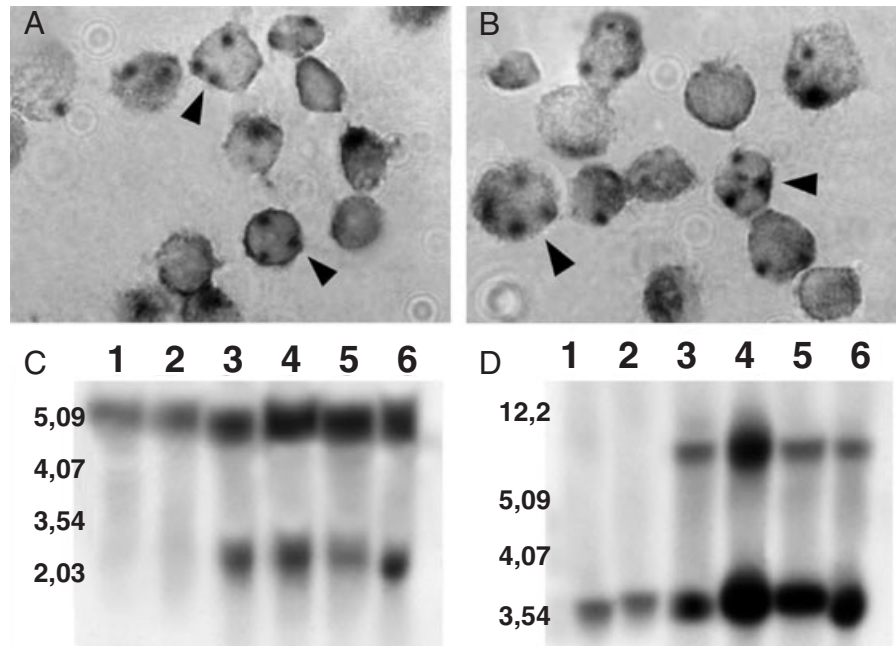


Figure 1. *In situ* PCR of bone marrow interphase nuclei of *Typanoctomys barrerae*: A, males; B, females. Southern blot of *T. barrerae* and *Pipanacoctomys aureus* genomic DNA cut to completion with C, *Bam*H1 and D, *Hind*III, separated on a 1% agarose gel. Lanes: 1, *O. mimax*; 2, *O. gliroides*; 3, *P. aureus* (male); 4, *T. barrerae* (female); 5, *T. barrerae* (male); 6, *T. barrerae* (male). Note only one band in lanes 1 and 2, whereas two bands are seen in lanes 3–6. The 145-bp *AR* fragment was used as probe in all experiments.

Southern blots of genomic DNA probed with its homologous Ty-AR145 gene fragment (Fig. 1C, D, lanes 3–5). Only one band was observed in the diploid controls (lanes 1 and 2), whereas two bands were observed in *T. barrerae*. Unexpectedly, *P. aureus* also showed two bands (lane 3).

MEIOSIS AND CYTOGENETIC ANALYSES

Male meiosis in *T. barrerae* is characterized by the strict formation of 51 bivalents that result in a perfect diploid-like chromosome pairing pattern (Fig. 2A). The typical end-to-end association of the XY bivalent (Hale, 1996) was observed in all cells. Complete pairing of the extra X chromosomes does not allow distinction from the other autosomes, assuming there are three X chromosomes as suggested by the *in situ* PCR. Male meiosis in *P. aureus* is also diploid-like, formed by 46 bivalents and end-to-end association of the sex chromosomes (Fig. 2B).

Bone-marrow metaphases of *P. aureus* have 92 biarmed chromosomes that include 25 pairs of metacentric to submetacentric and 19 pairs of subtelocentric chromosomes, most arranged in quadruplets according to shape and size similarity (Fig. 3A). Some quadruplets (formed by pairs 4–5, 12–13, 18–19) are

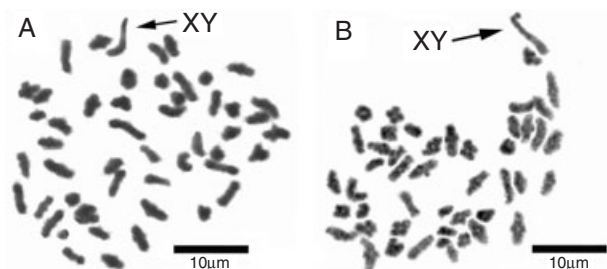


Figure 2. A, Diakinesis of a male *Typanoctomys barrerae* showing 51 bivalents. B, Diakinesis of a male *Pipanacoctomys aureus* showing 46 bivalents. Note the typical distal end-to-end association of the X–Y bivalent (arrow).

more uniform than others. The differing quadruplets (22–23, 37–38, 43–44) have paired similarly. Submetacentric pair 1 and the subtelocentric pair 28 cannot be arranged like the other chromosomes. The pair having a secondary constriction (SC) is the marker NOR chromosome in the Octodontidae (Spotorno *et al.*, 1995). As in *T. barrerae* (Gallardo *et al.*, 1999), the secondary constriction is present in only two chromosomes in *P. aureus*. The X chromosomes are tentatively assigned to the largest submetacentrics in the karyotype (Gallardo *et al.*, 1999). As in several other

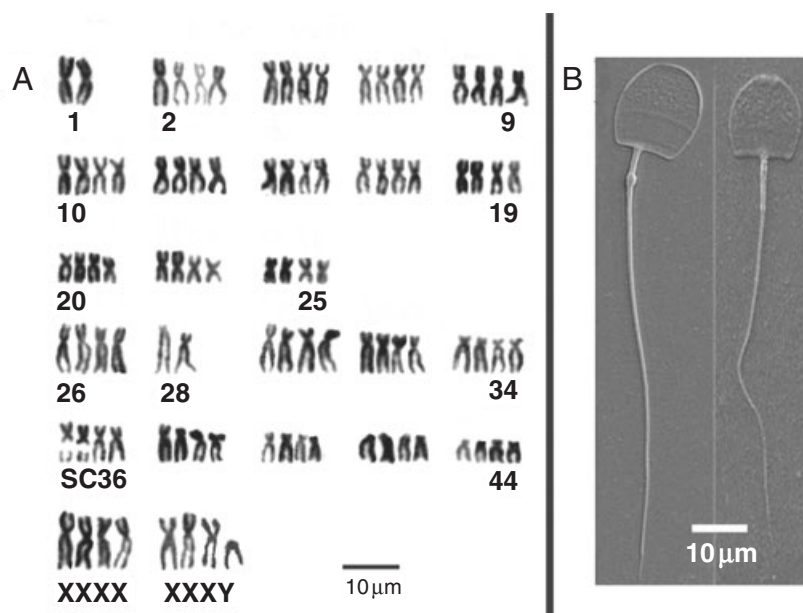


Figure 3. A, Karyotype of *Pipanacoctomys aureus* (Catamarca, Argentina) arranged as a tetraploid. Note that chromosome pairs 1, 28, and the marker chromosome (SC) pair do not form quadruplets. The identification of the sex chromosomes is tentative (see text for details). The Y is attributed to the only acrocentrics in the male karyotype. B, Scanning electron micrographs of the sperms of *Tympanoctomys barrerae* (left) and *P. aureus* (right).

octodontids (including *T. barrerae*) the Y chromosome of *P. aureus* is the only acrocentric in the karyotype (Gallardo, 1992; Gallardo *et al.*, 1999).

In *T. barrerae*, alteration of cell dimensions and increase in sperm head size are associated with its genome duplication (Gallardo *et al.*, 2002, 2003). Although smaller than the macrocephalic, paddle-like sperms of *T. barrerae* (14.2 µm in length, 13.7 µm in width, 0.4 µm in height; Fig. 3B), the sperms of *P. aureus* were also exceedingly large (11.2 ± 0.39 µm in length, 11.2 ± 0.40 µm in width). They are characterized by a spatulated head shape with a broad and flat lateral face (Fig. 3B). The flagellum attaches sub-medially to the truncated end of the head. The hood-like acrosome covers more than 50% of the head.

GENOME SIZE ESTIMATES

DNA content in *P. aureus* obtained from kidney cells indicated $2C = 15.34 \pm 0.67$ pg DNA whereas estimates of $1C = 7.18 \pm 0.56$ pg DNA were obtained from sperm cells. The mean genome size for 31 species of hystricognath rodents is 7.9 ± 1.9 pg DNA (Gallardo *et al.*, 2003)

PHYLOGENETIC RECONSTRUCTION

Regardless of the method and the model of evolution used, all analyses yielded the same tree topology.

Addition of the single *P. aureus* 12S rDNA sequence to the data matrix of Honeycutt *et al.* (2003) did not change overall relationships within the group but did suggest that *P. aureus* represents a sister group to *T. barrerae*. These taxa, together with *O. mimax* (with which they share a common ancestor), occupy a basal position among extant octodontids and form a well-supported sister group (100% bootstrap support) to the rest of the family (Fig. 4). Relationships among the other lineages are not completely resolved, but the basal placement of *O. gliroides* and the monophyly of the major genera (except for the clade including *Aconaemys* and *Spalacopus*) are well supported.

INTERSPECIFIC GENOMIC HYBRIDIZATION

Coinciding with the phylogenetic relationships, *Dra*-I digests of total genomic DNA probed with *P. aureus* revealed similar bands in the most closely related species. The strongest hybridization signals generated homologous bands between *T. barrerae*, *P. aureus* and *O. mimax* (Fig. 5). A reverse experiment, probing with *O. mimax*, also revealed high affinity, as if the samples were conspecifics (Thórsson *et al.*, 2001). Progressively fewer hybridization signals were obtained on *O. gliroides*, and few (if any) in the two outgroups used. No signal was obtained with the *M. musculus* control, corroborating the high divergence between Muridae and hystricognath rodents (Fig. 5). Other

experiments using *EcoRI* and *HpaII* resulted in similar banding homology (data not shown).

DISCUSSION

Genome size in *T. barrerae* and *P. aureus* suggest by itself an event of genome doubling because the mean DNA content for the hystricognath rodents is

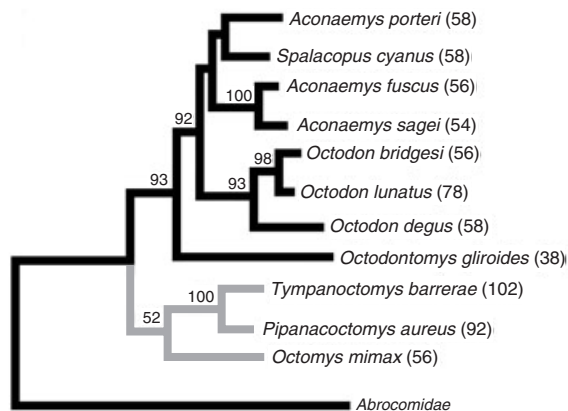


Figure 4. Mitochondrial phylogenetic relationships (12S rRNA) of the octodontid rodents. Bootstrap values are indicated at the nodes and chromosome numbers are given in parentheses. Grey lines refer to the desert-dwellers. Note the sister-group relationship between *Tympanoctomys barrerae* and *Pipanacoctomys aureus* within that clade.

7.9 ± 1.9 pg DNA (Gallardo *et al.*, 2003). Larger genome sizes can lead to larger nuclei and cell size. Such nucleotypic effects (Bennett, 2004 – this issue) have been reported in different cell lines and in the megacephalic sperms of *T. barrerae* (Gallardo *et al.*, 2003). Now they can be extended to the sperms of *P. aureus* (Fig. 3B).

Band number duplication in Southern hybridization experiments have demonstrated gene multiplicity in fungi (Carr & Shearer, 1998), monotremes (Lee *et al.*, 1999) and mice (Wang *et al.*, 2001). They have also confirmed ploidy level in cotton (Small & Wendel, 2000), bread wheat (Gornicki *et al.*, 1997) and cyprinid fishes (Nakao *et al.*, 2001). The two bands observed in the Southern blots of genomic DNA of *T. barrerae* and *P. aureus* indicate duplication of the *AR* locus (Fig. 1C, D). The maximum number of marks obtained by *in situ* PCR is interpreted to correspond to three X chromosomes [because the *AR* gene is X-linked (XXX \bar{Y})]. Likewise, the maximum number of discrete marks (four) in female nuclei of *T. barrerae* is interpreted to represent four X chromosomes (Fig. 1A, B).

Extensive long-term genomic changes are associated with polyploidy (Pikaard, 2001; Wolfe, 2001; Ainouche, Baumel & Salmon, 2004 – this issue). By being a marker chromosome in the Octodontidae (Spotorno *et al.*, 1995), the NOR chromosome is expected to occur in quadruplets. Nevertheless, only one NOR pair is seen in *T. barrerae* and *P. aureus*. Although this could

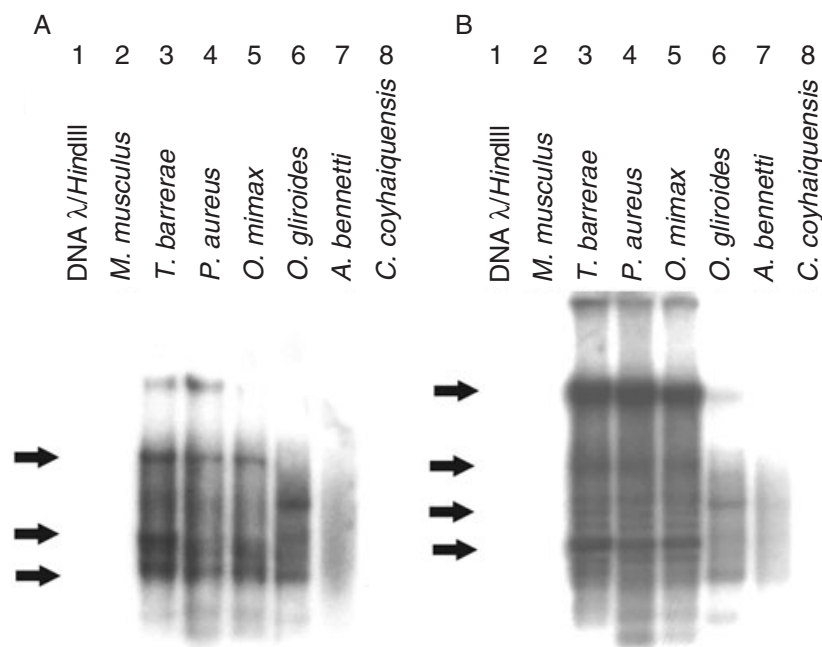


Figure 5. A, Southern genomic hybridization: total genomic DNA of *Pipanacoctomys aureus* (the probe) is hybridized to a Southern blot of *DraI*-digested genomic DNA of different octodontid species. B, Reverse experiment, but probing with *Octomys mimax*. Note the similarity with the reciprocal experiments. Lane 1, ladder. Lane 2, *Mus musculus*. Lanes 3–6 correspond to octodontids. Lanes 7 and 8 correspond to successive outgroups. Arrows indicate shared band homology.

suggest an unlikely hybridization event between an octodontid and a lineage lacking the NOR chromosome, nucleolar dominance may account for this peculiarity. Interestingly, studies in *Triticale* (the hybrid between wheat and rye) have demonstrated that wheat NORs are expressed and rye NORs are suppressed (Pikaard, 2001). Duplicated NOR sites in *T. barrerae* and *P. aureus* may have been either silenced or eliminated, such mechanisms can maintain a dosage-regulated gene expression (Osborn *et al.*, 2003).

Previous allozymic variation patterns (Köhler *et al.*, 2000), DNA annealing data (Gallardo & Kirsch, 2001) and sequence analyses (Honeycutt, Rowe & Gallardo, 2003) based on the examination of ten of the 13 extant species of octodontids identified the basal clade of the desert-dwellers to be *T. barrerae*, *O. mimax* and *O. gliroides*. A similar topology was recovered by our data, revealing a close sister relationship between *P. aureus* and *T. barrerae* with *O. mimax* their closest relative (Fig. 4).

New karyotypic data and the phylogenetic placement of *P. aureus* implies that a single lineage is insufficient to explain the origin of *T. barrerae* as previously thought (Gallardo *et al.*, 1999). Monophyly implies that both species having a duplicated genome size shared the same ancestors at about 6.5 Mya (Gallardo & Kirsch, 2001).

Assuming autotetraploidy from a 56-chromosome ancestor, the elimination of ten chromosomes in the karyotype of *T. barrerae* must be invoked. Otherwise, its origin must be traced back to a 51-chromosome ancestor, a most unlikely possibility. Likewise, a 20-chromosome elimination must be invoked to have occurred during the differentiation of *P. aureus* from its 56-chromosome lineage. Curiously, a symmetrical addition/subtraction of ten chromosomes connects both species. In addition to this peculiarity, rapid and total meiotic diploidization must be invoked to fit the meiotic behaviour of both taxa under autopolyploidization (Fig. 2A, B).

The correct pairing and segregation of chromosomes during meiosis is essential for genetic stability and organismal fertility (Stebbins, 1971). Nevertheless, the presence of additional chromosome sets in polyploids can affect the assortment of homologues, resulting in unbalanced gametes (Soltis & Soltis, 1995, 1999; Soltis *et al.*, 2004 – this issue). Assuming that *T. barrerae* and *P. aureus* are tetraploids, strict bivalent formation suggests an allopolyploid origin or rapid genome divergence in autopolyploid lineages (Levy & Feldman, 2004 – this issue). Interestingly, by adding ten chromosomes to *P. aureus*, the chromosome number of *T. barrerae* is obtained. The chromosomal difference between both descendant species relative to their ancestral lineage requires an explanation. Although identifying species contributing to

allopolyploids is often very difficult, some insights may be gained by considering the phylogenetic relationships and the chromosome numbers of potential parental contributors relative to the extant tetraploids. Apparently, two ancestral lineages allied to *O. mimax* (but differing in chromosome number between them) may have donated its 92-chromosome complement. This is supported by the strict bivalent formation observed in meiotic plates and that it is impossible to arrange all chromosomes of the karyotype of *P. aureus* in quadruplets (Fig. 3). By the same token, the karyotype of *T. barrerae* can be derived by assuming hybridization (and backcrossing) between tetraploid *P. aureus* and diploid *O. mimax*, provided that non-fused gametes were produced in the latter species. Thus, the diploid-like meiotic pairing in *P. aureus* and *T. barrerae* may be associated with correct chromosome segregation (segmental polyploids?) as with the diploid-like meiotic behaviour and homologous pairing of allopolyploid wheat (Liu *et al.*, 1998; Moore, 2002). Although gene redundancy could also be explained by gene duplications, allopolyploidy provides a better explanation for all the evidence gathered because it does not require *ad hoc* hypotheses nor conflicts with the data sets.

If a species is a natural hybrid, it is expected to contain DNA bands specific to its parental species, as demonstrated in birch trees (Thórsson *et al.*, 2001) and grass species (Anamthawat-Jónsson *et al.*, 1990; Pašakinskiene *et al.*, 1998). Genomic Southern hybridization experiments are consistent with the notion that *Octomys mimax* is involved in the genesis of both *P. aureus* and *T. barrerae*. The high overall DNA similarity, shared band homology and matching chromosome numbers between *O. mimax* and the descendant tetraploid species support introgressive hybridization (Fig. 4). If this model holds true, three genomes concurred in the genesis of *T. barrerae*. Further cytogenetic and meiotic analyses will be needed to corroborate these tenets.

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