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Molecular cytogenetics and allotetraploidy in the red vizcacha rat, *Tympanoctomys barrerae* (Rodentia, Octodontidae)

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Abstract

The theoretical impossibility of polyploidy in mammals was overturned by the discovery of tetraploidy in the red vizcacha rat, *Tympanoctomys barrerae* ($2n = 102$). As a consequence of genome duplication, remarkably increased cell dimensions are observed in the spermatozoa and in different somatic cell lines of this species. Locus duplication had been previously demonstrated by in situ PCR and Southern blot analysis of single-copy genes. Here, we corroborate duplication of loci in multiple-copy (major rDNAs) and single-copy (*Hoxc8*) genes by fluorescence in situ hybridization. We also demonstrate that nucleolar dominance, a large-scale epigenetic silencing phenomenon characteristic of allopolyploids, explains the presence of only one Ag-NOR chromosome pair in *T. barrerae*. Nucleolar dominance, together with the chromosomal heteromorphism detected in the G-banding pattern and synaptonemal complexes of the species' diploid-like meiosis, consistently indicates allotetraploidy. Allotetraploidization can coherently explain the peculiarities of gene silencing, cell dimensions, and karyotypic features of *T. barrerae* that remain unexplained by assuming diploidy and a large genome size attained by the dispersion of repetitive sequences.

Keywords: Allotetraploidy; NORs; Nucleolar dominance; Red vizcacha rat; *Tympanoctomys barrerae*

Polyploidy has played a significant role in the hybrid speciation and adaptive radiation of flowering plants [1] but has been considered irrelevant to mammalian speciation due to severe disruptions in the sex-determination system and dosage compensation mechanism [2,3]. For instance, triploid and tetraploid humans are aborted or die soon after birth due to multiple internal and external malformations, including gonadal dysgenesis [4,5].

The discovery of tetraploidy in the red vizcacha rat, *Tympanoctomys barrerae* ($2C = 16.80 \pm 0.50$ pg DNA) overturned this tenet [6] but the origin of its completely biarmed, 102-chromosome karyotype remained obscure, since no combination of diploid chromosome numbers in extant family members could explain the derivation of its complement [7]. The unexpected discovery of tetraploidy in another octodontid species, *Pipanacoctomys aureus* [8], and its sister relationship to *T. barrerae* have helped to predict the past reticulate history of these species. Like *T. barrerae*, *P. aureus* ($2n = 92$) has a genome size that is twice that of its close relatives ($2C = 15.34 \pm 0.67$ pg DNA), and it has a totally biarmed autosomal set, except for the Y chromosome [7]. The acrocentric Y chromosome has been corroborated in all octodontid species reported [9,10].

The dramatic increase in cell size (*gigas* effect) produced by the triggering action of regulatory mechanisms is the most widespread phenotypic effect reported in polyploid plants [1], but is not common in animals. Interestingly, the *gigas* effect is observed in the significantly larger somatic cells [12] and record sperm head dimensions of *T. barrerae* and *P. aureus* [7]. This effect can be achieved by increasing the length of the cell cycle through repression of the G1 cyclins according to ploidy level [11]. Cell binucleation is another cellular effect associated with the physiological polyploidization that prevails in human hepatocytes. This feature has been also recorded in 26% of luteal bodies of *T. barrerae* but not in the diploid control, *Spalacopus cyanus* [13].

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Ribosomal RNA (rRNA) gene transcription accounts for most of the nuclear transcriptional dynamics in actively growing eukaryotic cells. The secondary constriction of chromosomes (SC), also detected by silver impregnation (Ag-NOR) at the nucleolus organizer region (NOR), is the indicator of this activity regardless of the rDNA site number [14].

The constancy of only one NOR chromosome pair in all octodontids, no matter what their ploidy level [10], was advanced to suggest chromosome elimination to regulate effective gene dosage in tetraploid *T. barrerae* [6]. Nevertheless, a recent report based on chromosome painting considers the single NOR chromosome pair in the red vizcacha rat as evidence of diploidy and claims that the accumulation of repetitive sequences explains its large genome size [15]. Interestingly, the incongruence between total and active rDNA sites is explained by nucleolar dominance in allopolyploids and diploid hybrids [14,16] in that the NOR loci derived from one progenitor are transcriptionally active while the ones derived from the other are silenced [17–19].

The controversy over the ploidy level of *T. barrerae* is most relevant to assess a novel mechanism of genome evolution in mammals and to understand fully the karyotypic evolution of the octodontids [7]. This prompted us to test the hypothesis of diploidy by using fluorescence in situ hybridization (FISH) with major rDNA probes (18S, 5.8S, and 28S) to metaphase spreads of *T. barrerae* and its diploid relatives. We also extend the direct evidence that supports tetraploidy by using the single-copy gene *Hoxc8* as probe. G-banding patterns and the meiotic configuration of surface-spread synaptonemal complexes of *T. barrerae* are also presented to gain a better understanding of chromosomal segregation and its bearing on the origin of this peculiar rodent species.

Results

The rDNA probe detected one hybridization signal in each of two homologous chromosomes (one locus) in *Octodon degus*, *Octomys mimax*, and *S. cyanus*, as expected from their diploid condition (Figs. 1A–1C). However, one signal in each of four different chromosomes (two loci) was detected in 80% of the mitotic plates of *T. barrerae*, as would be expected from its tetraploid condition (Fig. 1D). The *Hoxc8* probe detected one hybridization signal in each of two homologous chromosomes of *S. cyanus* and *Octom. mimax* (Figs. 2A and 2B), whereas it detected one signal in four different chromosomes (two loci) in metaphase spreads of *T. barrerae* (Fig. 2C). By contrast, one set of transcriptionally active rDNA gene clusters was invariably revealed by Ag-NOR in diploid *Octod. degus*, *S. cyanus*, and *Octom. mimax*, as well as in tetraploid *T. barrerae* (Fig. 3).

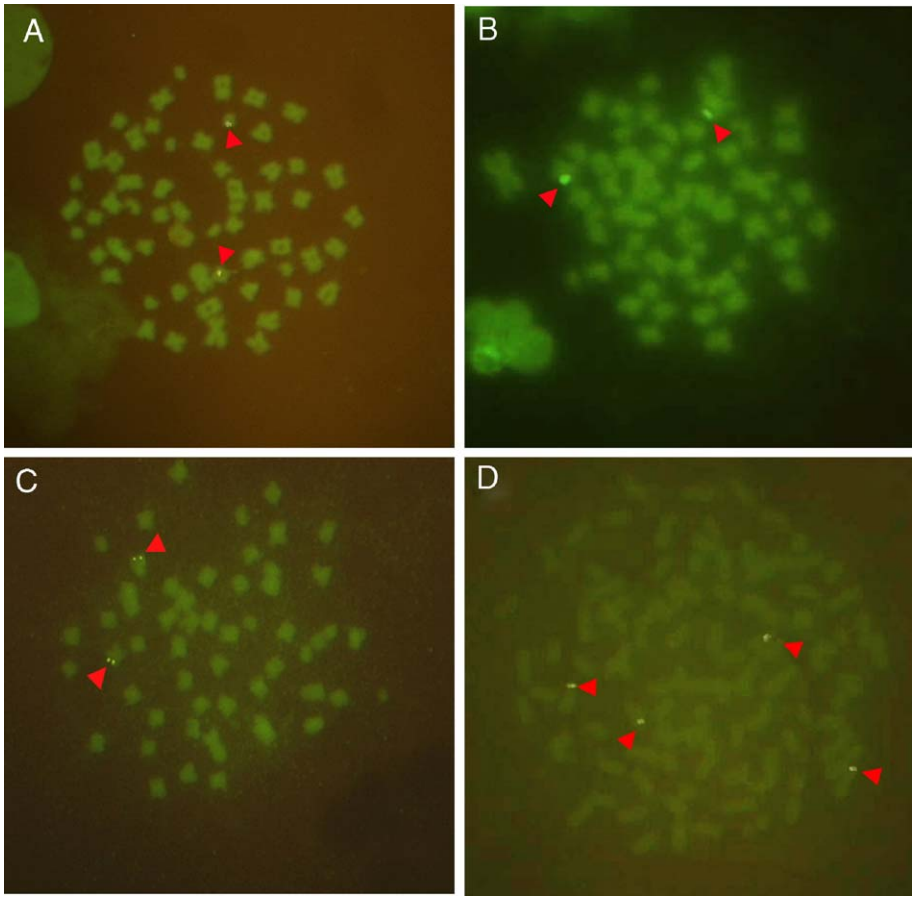

Fig. 1. FISH using rDNA as probe in diploid and tetraploid octodontid rodents. Two hybridization spots were visualized on the karyotype of (A) *Octod. degus*, (B) *S. cyanus*, and (C) *Octom. mimax*. (D) Four hybridization signals were detected on the karyotype of *T. barrerae*.

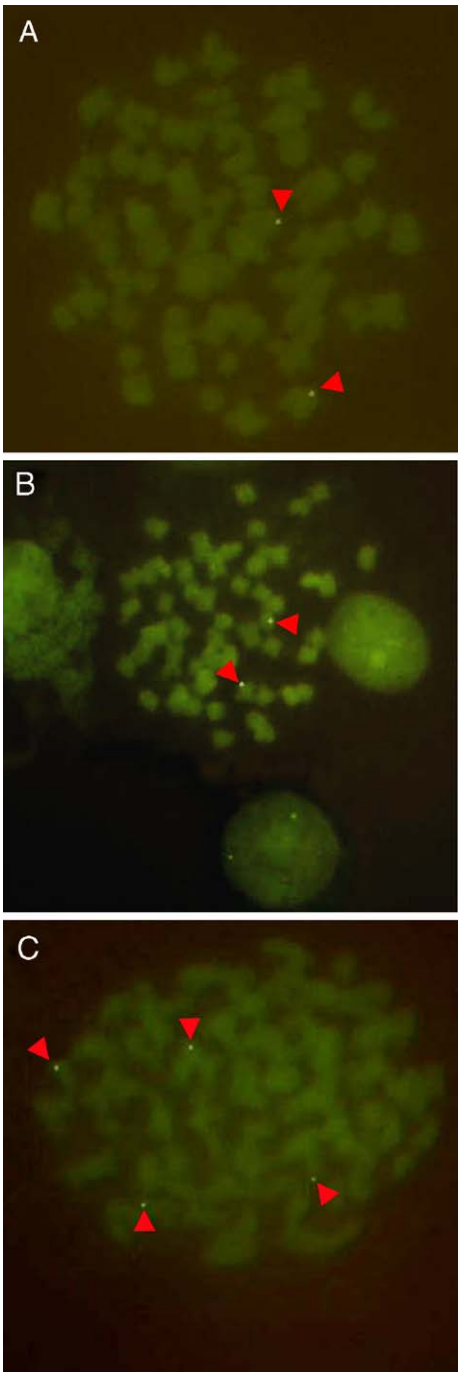

Fig. 2. FISH signal images of diploid and tetraploid octodontid rodents probed with *Hoxc8*. (A) *S. cyanus*. (B) *Octom. mimax*. (C) *T. barrerae*.

The male meiosis of *T. barrerae* is totally diploid-like and consists of 51 bivalents (Fig. 4). Interestingly, marked heteromorphic meiotic pairing of 12–15 synaptonemal complexes is evident due to length differences between homologues. This heteromorphism is observed during pachytene but is equalized during diakinesis by coiling of the longer element around the shorter one. A single, heteromorphic X–Y bivalent showing the typical end-to-end association is also observed (Fig. 4, inset).

The G-banding revealed marked differences in band width and chromosome length between homologues of *T. barrerae* (Fig. 5). Due to this remarkable heteromorphism, most elements were arranged in quadruplets that share either disomic or trisomic similarity, whereas other chromosomes can be grouped only in pairs. Homologues of pairs 2, 4, 5, 6, 8, 10, 14, 20, 28, 40, 43, and 45 differ in overall size. Disomic similarity is observed in quadruplets 3 and 4, 7 and 8, 9 and 10, 15 and 16, 27 and 28, 37 and 38, and 46 and 47. Trisomic similarity is evident in quadruplets containing pairs 2, 6, 14, 16, 43, 45, and 49. Elements 35, 36, and 41 are arranged in pairs due to their peculiar morphology and banding pattern. Pair 41 is the active NOR element, having an interstitial SC in the long arm (Fig. 5). Identification of the inactive NOR chromosome pair is not possible with this technique. Based on previous reports, the X chromosomes are considered to be the largest elements, while the Y chromosomes are monoarmed and are observed only in male karyotypes [6,7,20].

Discussion

Locus duplication was previously demonstrated by in situ PCR and Southern blot analysis of the sex-linked androgen receptor gene in *T. barrerae* [7]. Here, further locus duplication in the *Hoxc8* gene is demonstrated by molecular cytogenetics. One hybridization signal was observed on each member of one homologous chromosome pair in diploid octodontid species, whereas two chromosome pairs each exhibited two hybridization signals in *T. barrerae* (Figs. 1 and 2). In addition, nucleolar dominance demonstrates that epigenetic silencing rather than diploidy explains the presence of only one Ag-NOR chromosome pair in the red vizcacha rat.

NOR loci have been satisfactorily used to substantiate genome duplication [21,22] and to corroborate the hybrid origin of the polyploid plant genera *Lilium* [17], *Citrus* [23], and *Nicotiana* [24] via control mechanisms that regulate gene silencing [16]. The transcriptional activity of this locus is detected by the specific binding affinity of silver to argirophyllic proteins associated with the RNA Pol I machinery complex [25].

Nucleolar dominance is a well-known regulatory phenomenon affecting the active number of NOR loci in allopolyploids and diploid hybrids [14]. This silencing mechanism depends on an epigenetic switch that in concert with promoter methylation and histone modifications results in a self-reinforcing repression cycle of the rRNA genes [26]. In *Triticale* (a hybrid between wheat and rye), the wheat NORs are active and the rye NORs are silenced [27]. The generality of this epigenetic mechanism is further exemplified in synthetic and natural allopolyploids like *Arabidopsis* [14,28], *Brassica* [29], *Glycine* [30], *Gossypium* [31], *Nicotiana* [16], and *Triticum* [32]. Nucleolar dominance is also observed in hybrid diploid mammals. In the mule, the active NOR locus is derived from the donkey progenitor, whereas the horse-derived counterpart is silenced [33]. In phyllotine rodents (*Phyllotis darwini* × *Ph. magister*), the active NOR is derived from *Ph. darwini* while the *Ph. magister*-derived NOR is silenced [34,35]. Further evidence of nucleolar dominance in hybrid animals is provided by *Drosophila* [36]

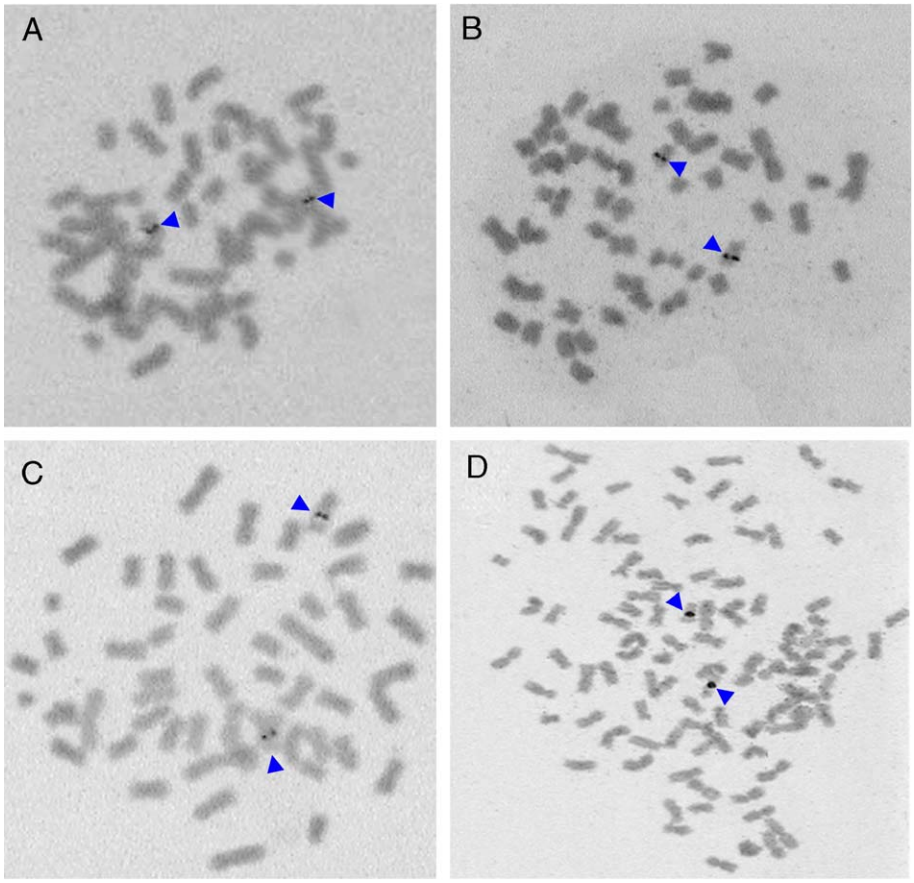

Fig. 3. Ag-NOR staining in diploid and tetraploid octodontid rodents. This procedure allows the identification of one active NOR chromosome pair in each rodent species, regardless of ploidy level. (A) *Octod. degus*. (B) *S. cyanus*. (C) *Octom. mimax*. (D) *T. barrerae*.

and *Xenopus* [37]. Interestingly, the exact epigenetic silencing in allopolyploids contrasts with the variability of active NORs in autopolyploids. The presence of one, two, three, or more active nucleoli in different cells of the autopolyploid plant *Silene latifolia* [38] and the frog *Odontophrynus americanus* [39,40] denotes the failure of nucleolar dominance to discriminate among the NOR loci when the same genome is duplicated. Keeping in mind that all octodontids exhibit only one positive Ag-NOR chromosome pair regardless of ploidy level [10], our Ag-NOR and FISH results in the diploid octodontids are consistent with each other and with previous data. In contrast, the double number of labeled chromosomes detected by FISH relative to positive Ag-NOR signals in *T. barrerae* falsifies the notions of chromosome elimination [6] and diploidy [15] since the transcriptional silencing of one parental NOR locus implies allotetraploidy [16,26].

Meiotic pairing is also a good indicator of origin [41,42] since autopolyploid frogs [39,40] and plants show varying degrees of multivalent formation and polysomic segregation [38]. In contraposition, allopolyploid taxa exhibit bivalent formation and disomic segregation to restore fertility, as in diploid organisms [43,44]. This cytological diploidization has been reported in corn (*Zea mays*) [45,46] and *Arabidopsis* [44], which have been proven to be allotetraploids by molecular and cytogenetic analyses. Meiotic diploidization is also observed in wheat [47], tobacco, cotton [48], and the *Polystichum* fern

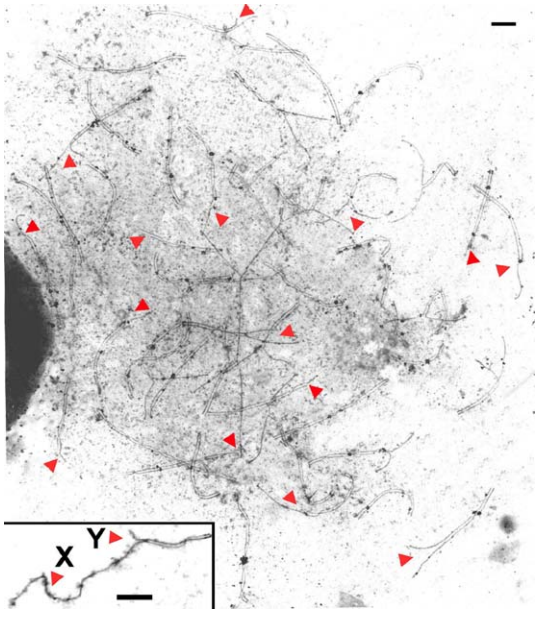

Fig. 4. Whole-cell complement synaptonemal complexes in surface-spread midpachytene nuclei from *T. barrerae*. The typical distal end-to-end association of the X–Y bivalent from another cell is inset. Note the monoarmed morphology of the Y. Red arrows indicate length differences of lateral elements. Bar, 2.5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

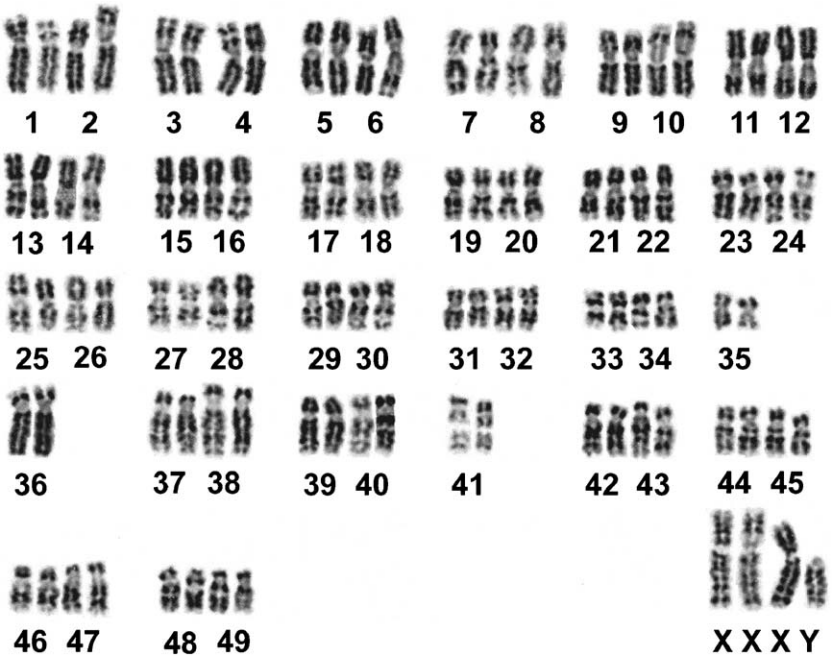

Fig. 5. G-bands of *T. barrerae*'s chromosomes arranged in quadruplets. Disomic or trisomic similarity is observed in some quadruplets. Pair 41 is the NOR pair, having a secondary constriction in the long arm. The X chromosomes are among the largest elements, and Y is the only acrocentric in the karyotype.

complex containing allotetraploid and allo-octoploid species [49]. Furthermore, the deca-allopolyploid clams *Pisidium* [50] and allopolyploid fern genera *Asplenium* [51] and *Equisettum* show strict bivalent formation and disomic segregation [52]. Thus, the diploid-like meiotic behavior, disomic segregation of allozymic loci [53], and chromosomal disparities detected in different specimens of *T. barrerae* are not evidence per se of diploidy but of allotetraploidy [15]. Indeed, the chromosomal heteromorphism detected by the G-bands of the red vizcacha rat are equivalent in kind and degree to those reported for interspecific hybrids within the rodent genera *Uromys* [54], *Melomys* [55], and *Phyllotis* [34,35]. Most probably, the diploid-like cytological features of *T. barrerae* together with the epigenetic modifications exemplified by the gene silencing of one NOR locus are likely the leading factors promoting genetic diploidization in this tetraploid taxon [44].

The phylogeny of the octodontids is characterized by a basal dichotomy in which the monophyletic group of desert specialists from Argentina is formed by the sister-group relationship between *T. barrerae* and *P. aureus*. *Octom. mimax* is basal to this clade and sister to them [7,56]. Previously reported overall DNA homology by intergenomic Southern hybridization of these species and their matching chromosome numbers was used to advance the notion of allotetraploidy in the red vizcacha rat [7]. The results presented here are congruent with one another and with previous data. Their consistency further favors the hypothesis of allopolyploidy over diploidy.

The claim that *T. barrerae* is diploid [15] fails to explain the phylogenetic relationships of the tetraploid octodontids and their bearings upon the evolution of a 102-chromosome descendant from a 56-chromosome ancestor [7]. The crux of this diploid hypothesis is that the ancestral and descendant taxa have all-biarmed karyotypes with exclusively pericentromeric heterochromatin [6,9,10,20]. Consequently, if multiple Robertsonian fissions are invoked to explain the karyotypic evolution of *T. barrerae*, subsequent secondary growth of heterochromatic arms, as in the paradigmatic example of the *Thomomys bottae* pocket gopher [57], would be needed to attain its biarmed chromosome condition. Alternatively, the highly improbable de novo formation of 50 centromeres would be required to fit the data at hand. Intriguingly, no heterochromatic arms but disperse (C-negative) heterochromatin is reported by Svartman et al., and no explanation for such a contradiction is offered [15]. Likewise, the misidentification of the only acrocentric element (Y chromosome) in *Octod. degus* and *T. barrerae* contradicts previous reports [9,10,58] and further obscures the cytogenetic conclusions sustaining the claim of diploidy.

The dramatic *gigas* effect observed in the spermatozoa of *T. barrerae* and *P. aureus* [6,7,13,59] demands an explanation, also lacking in the proposition of diploidy by Svartman et al. [15]. The larger spore size in polyploid ferns [49] and in diploid spermatozoa of rabbits [60] and bovines [61] reveals that gamete dimensions are triggered by gene interactions operating at the expression level through the gene-regulation networks [44]. Likewise, the increased cell dimensions observed in polyploid tissues of plants [1], nematodes [62], *Drosophila* [63], and yeasts [11] result from the complexities of regulatory signaling pathways associated with ploidy level; not from the dispersion of repetitive sequences.

Genome size increase through a quantum shift in DNA content in the octodontids represents a novel mechanism of genome evolution in mammals [7,12]. Although the mechanism to increase the DNA content is clearly distinct and independent of the chromosomal rearrangements involved in the karyotypic evolution of the octodontids [9], the saltational nature of both phenomena has led to confusion. We agree with Svartman et al.

[15] that the 78-chromosome *Octodon lunatus* is midway in a numerical progression from the lowest 38-chromosome *Octodontomys gliroides* to the 102-chromosome *T. barrerae*. Nevertheless, this variation is not relevant to the issue of quantum genome size shifts since the 8.8 pg DNA of *Octod. lunatus* and the 15.3 pg DNA of the 92-chromosome *P. aureus* corresponds to diploid and tetraploid genome size peaks, respectively [12].

The robust phylogeny of the octodontids has provided an interpretative framework for understanding the evolutionary patterns that allows drawing inferences about the process that explain the peculiar character association of record sperm dimensions, genome size duplication, and largest chromosome number in *T. barrerae* [7,56]. While allotetraploidization discloses a general congruency among independent molecular and cytological data sets in accordance with the systematic relationships of the octodontids, the claim of diploidy has limited explanatory capabilities and lacks viable alternative explanations. This is evident in its failure to explain both the *gigas* effect of the red vizcacha rat and the derivation of its all-biarmed karyotype. By extension, the same limitations apply to *P. aureus*. Due to phylogenetic congruency among diverse biological features and having met more explanatory challenges, allotetraploidy remains the most parsimonious and robust proposition for the origin of this unusual rodent species.

Materials and methods

Fluorescence in situ hybridization

Metaphase cells of the octodontids *Octom. mimax* ($n = 4$), *Octod. degus* ($n = 1$), *S. cyanus* ($n = 4$), and *T. barrerae* ($n = 5$) were prepared using standard procedures [64]. FISH was performed using a 5-kb ribosomal probe from the carp *Cyprinus carpius* containing the major rRNA gene family. In addition, a 20-kb probe containing the linked genes *Hoxc8* and *Hoxc9* was obtained by screening the genomic library of *T. barrerae*. We refer to this in the text as the *Hoxc8* probe. To detect loci with the greatest possible sensitivity, single-label rather than dual-label FISH was used. Biotin-labeled probes were prepared by nick translation using Bio-14–dATP, following the manufacturer's specifications (Invitrogen). Slides were pretreated with 1 μg RNase A/ml of 2× SSC at 37°C for 60 min, washed three times in 2× SSC at 20°C for 5 min, and hydrolyzed with 10 mM HCl 37°C for 2 min. Then, the slides were treated with 100 μl pepsin (5 mg/ml in 10 mM HCl) at 37°C for 10 min and washed three times in 2× SSC. The samples were denatured at 65°C in 70% formamide/2× SSC for 3 min, quenched in ice-cold ethanol, and dehydrated in a cold ethanol series for 3 min. Probes (100 ng) were mixed with a hybridization solution containing 50% formamide, 10% dextran sulfate, 0.1% SDS in 2× SSC (pH 7.0) and denatured at 100°C for 15 min. Hybridization was carried out in a moist chamber at 37°C for 72 h. Posthybridization washes at 37°C consisted of 50% formamide/2× SSC followed by 3 × 3-min washes in 2× SSC and 3 × 5-min washes in 4× SSC/0.1% Tween 20. Probes were detected after 1-h incubation with avidin–FITC (Sigma) in a moist chamber and washed for 5 min in PBT at 37°C [65]. Chromosomes were counterstained with DAPI and propidium iodide added to the antifade solution (Vectashield). Between 15 and 30 mitotic plates of each species were examined with an epifluorescence microscope (Axiolab; Carl Zeiss) for data consistency. Images were digitally captured and contrast-enhanced using PhotoShop 7.0.

G-bands of *T. barrerae* were obtained from fibroblast cultures of lung tissue from an adult male (MHG 1755) from El Nihuil (Mendoza Province, Argentina). Bands were induced by trypsin treatment [64]. Chromosomes were arranged by quadruplets of decreasing order (metacentric–submetacentric first) and numbered by pairs.

Microspreading

To increase sample size and to avoid drawing conclusions from a single specimen, a different adult male of *T. barrerae* (IEEUACH 7097) from the same locality was processed for meiotic analyses. Testes were processed to obtain spermatocyte nuclei for analysis of whole-cell complements of synaptonemal complexes, according to the spreading protocol for rodents [66]. A drop of the cell suspension was placed onto an acetate-covered microscope slide along with two drops of swelling solution composed of 10 μl 0.1 M EDTA, 10 μl 0.06 M phosphate buffer, 3 μl 1% Triton X-100, and 77 μl distilled water. Final pH was adjusted to 7.5 with 1 N NaOH. Cells were fixed after 12 min in a solution containing 4% paraformaldehyde and 1.7% sucrose (pH 8.9). Slides were dried at 60°C for 6 h and then washed for 5 min in distilled water. Staining was performed with 50% $AgNO_3$ (pH 3.2). A total of 26 sex bivalents and 31 complete nuclei were photographed and analyzed. Observations were performed with a Jeol JEM 1010 TEM. Zygotene and pachytene stages were classified according to the criteria described by Greenbaum et al. [67] and Giménez et al. [68].

Acknowledgments

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