Chromosome Res (2012) 20:259–268
DOI 10.1007/s10577-011-9261-0

Multiple independent evolutionary losses of XY pairing at meiosis in the grey voles

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Received: 5 October 2011 / Revised and Accepted: 23 November 2011 / Published online: 13 December 2011

Abstract In many eutherian mammals, X–Y chromosome pairing and recombination is required for meiotic progression and correct sex chromosome disjunction. Arvicoline rodents present a notable exception to this meiotic rule, with multiple species possessing asynaptic sex chromosomes. Most asynaptic vole species belong to the genus *Microtus* sensu lato. However, many of the species both inside and outside the genus *Microtus* display normal X–Y synapsis at meiosis. These observations suggest that the synaptic condition was present in the common ancestor of all voles, but gaps in current taxonomic sampling across the arvicoline phylogeny prevent identification of the lineage(s) along which the asynaptic state arose. In this study, we use electron and immunofluorescent microscopy to assess heterogametic sex chromosome pairing in 12 additional arvicoline species. Our sample includes ten species of the tribe Microtini and two species of the tribe Lagurini. This increased breadth of sampling allowed us to identify asynaptic species in each major Microtine lineage. Evidently, the ability of the sex chromosomes to pair and recombine in male meiosis has been independently lost at least three times during the evolution of Microtine rodents. These results suggest a lack of evolutionary constraint on X–Y synapsis in Microtini, hinting at the presence of alternative molecular mechanisms for sex chromosome segregation in this large mammalian tribe.

Keywords meiosis · recombination · synapsis · synaptonemal complex · pseudoautosomal region · MLH1 · SCP3 · vole · Arvicolinae

Responsible Editor: Herbert Macgregor.

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Abbreviations

BSA	Bovine serum albumin
Cy3	Orange fluorescing cyanine
DAPI	4′-6-Diamidino-2-phenylindole
EM	Electron microscopy
FITC	Fluorescein isothiocyanate
FM	Fluorescent microscopy
MLH1	Homolog of prokaryotic mutL 1 mismatch repair protein
SC	Synaptonemal complex
SCP3	Synaptonemal complex protein 3

Introduction

The segregation of homologous chromosomes into daughter cells at meiosis depends on the timely and precise execution of homolog pairing, synapsis, and recombination. Unpaired chromosomes and chromosomes that fail to undergo recombination can activate early meiotic checkpoints, leading to arrest of the meiotic cell cycle or programmed cell death. Defects in meiotic chromosome pairing and recombination are established mechanisms of infertility in humans (Hassold and Hunt 2001).

Mammalian heterogametic sex chromosomes present a notable deviation from this meiotic routine. The X and Y chromosomes are largely non-homologous, with only short terminal regions of sequence homology. Pairing and recombination in these pseudoautosomal regions (PAR) is required for meiotic progression and proper disjunction of the sex chromosomes in many mammalian species (Mangs and Morris 2007). Structural mutations in the PAR have been directly linked to spermatogenic failure in humans and mice (Burgoyne et al. 1992; Mohandas et al. 1992), and divergence in the PAR between isolated populations could provide an intrinsic genetic barrier to hybridization, driving the formation of new species.

Despite the clear link between X–Y pairing and meiotic progression in many mammals, there are a handful of exceptional species. For example, in marsupials and several rodent species, the X and Y chromosomes remain unpaired throughout meiosis (Deakin et al. 2010). In these species, the sex chromosomes are sequestered into a dense sex body, but they never synapse or recombine (Page et al. 2006). Curiously, most of the mammalian species with asynaptic sex chromosomes also possess unusually large sex chromosomes that contain large blocks of constitutive heterochromatin (Marchal et al. 2004). Whether this unique karyotypic feature is functionally related to sex chromosome behavior at meiosis remains to be elucidated.

Arvicoline rodents present an especially powerful test case for studying X–Y pairing and recombination at meiosis. Arvicolinae is a speciose mammalian subfamily in which many species with asynaptic sex chromosomes have been found (Fig. 1). The availability of closely related species that follow distinct meiotic paradigms provides a unique model system for dissecting the molecular and evolutionary basis for the loss of sex chromosome pairing and recombination.

Most of the voles with asynaptic sex chromosomes belong to the genus *Microtus* sensu lato. All species of Palearctic subgenera *Microtus*, *Agricola*, *Sumeriomys*, *Iberomys*, *Terricola*, and *Blanfordimys* studied so far are asynaptic (Ashley et al. 1989, 1990; Borodin et al. 1995; Carnero et al. 1991; Jimenez et al. 1991; Rovatsos et al. 2008; Wolf et al. 1988). Normal X–Y synapsis has been observed in all studied species of East Asian subgenera *Pallasiinus* (Ashley and Fredga 1994; Borodin et al. 1997, 1995; Mekada et al. 2001) and *Alexandromys* (Borodin et al. 2011). Synaptic sex chromosomes have also been found in arvicoline species closely related to the genus *Microtus*, such as *Neodon sikimensis* (Mekada et al. 2002) and *Chionomys nivalis* (Megias-Nogales et al. 2003), and in the distantly related species *Arvicola terrestris* and *Arvicola sapidus* (Borodin et al. 1995; Megias-Nogales et al. 2003), *Ellobius talpinus* (Bogdanov et al. 1986), and six species of the genus *Myodes* (Ashley and Fredga 1994; Borodin et al. 1995; Iwasa et al. 1999).

Together, these patterns suggest that the synaptic condition is the ancestral state in arvicoline rodents. Apparently, it has been retained in the East Asian lineage of the genus *Microtus*, but lost in the other lineage (or lineages) of this genus (Borodin et al. 2011). However, the synaptic patterns of several lineages in the genus *Microtus* sensu lato remain unknown. Determining the XY pairing status in Nearctic voles, which are considered a sister group to the Asian and Palearctic lineages (Conroy and Cook 2000), and in some early branches of the tribe *Microtini*, such as *Stenocranius* and *Lasiopodomys* (Abramson et al. 2009), could help shed light on the evolutionary origin(s) of XY asynapsis on the arvicoline phylogeny.

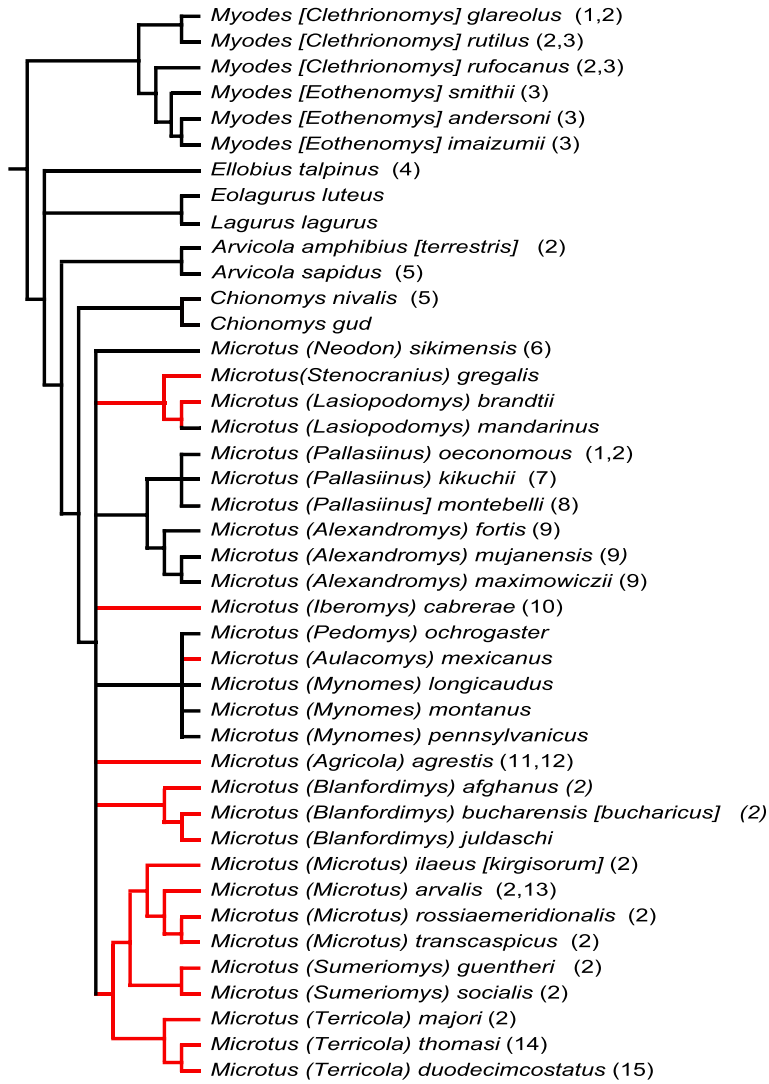

Fig. 1 Informal supertree of the arvicoline rodents with a known pattern of X–Y pairing. The tree is based on published molecular phylogenies (Abramson et al. 2009; Bannikova et al. 2009, 2010; Conroy and Cook 2000; Jaarola et al. 2004). Nodes receiving less than moderate support are reduced to polytomies. Species names are given according to recent publications and the checklist (Wilson and Reeder 2005). The subgenus names are given in *brackets*; the names used in the original publications are given in *square brackets*. *Numbers in parentheses* indicate the source of information about X–Y synaptic condition: *1* Ashley and Fredga 1994; *2* Borodin et al. 1995; *3* Iwasa et al. 1999; *4* Bogdanov et al. 1986; *5* Megias-Nogales et al. 2003; *6* Mekada et al. 2002; *7* Mekada et al. 2001; *8* Borodin et al. 1997; *9* Borodin et al. 2011; *10* Jimenez et al. 1991; *11* Ashley et al. 1990; *12* Wolf et al. 1988; *13* Ashley et al. 1989; *14* Rovatsos et al. 2008; *15* Carnero et al. 1991. *Black lines* indicate synaptic species; *red lines*, asynaptic

Toward this goal, we applied electron and immunofluorescent microscopy of surface-spread synaptonemal complexes (SC) to analyze sex chromosome pairing in ten species of the tribe *Microtini* and two species of the tribe *Lagurini*. We identify several independent losses of sex chromosome pairing across the vole phylogeny, a result that hints at the presence of an as yet uncharacterized mechanism for segregating unpaired, achiasmate sex chromosomes in these species.

Material and methods

A total of 23 adult males from 12 species were examined in this analysis. Sampling locations are provided in Table 1.

Our sample includes five species of the Nearctic grey voles (*Microtus (Mynomes) longicaudus* Merriam 1888, *Microtus (Mynomes) montanus* Peale, 1848, *Microtus (Mynomes) pennsylvanicus* Ord, 1815, *Microtus (Pedomys) ochrogaster* Wagner, 1842., and *Microtus (Aulacomys) mexicanus* Saussure, 1861); the widely distributed *Microtus (Stenocranius) gregalis* Pallas, 1779; two East Asian species (*Lasiopodomys brandtii* Radde, 1861 and *Lasiopodomys mandarinus* Milne-Edwards, 1871); and the Central Asian species *Microtus juldaschi* (Severtzov, 1879). *M. juldaschi* had formerly been assigned to the genus *Neodon* (Musser and Carleton 2005) in which one synaptic species, *N. sikimensis*, has already been described (Mekada et al. 2002). However, *M. juldaschi* was recently assigned to the genus *Microtus* (*Blanfordimys*) based on an analysis of

Table 1 Synapsis between X and Y chromosomes in the species studied

Species	Sampling locality	No. of animals	No. of cells FM	No. of cells EM	Percent of cells containing X and Y paired at pachytene
Eolagurus luteus	Volgograd Distr., Russia	1	50		94
Lagurus lagurus	East Kazakhstan Distr., Kazakhstan	1	25		86
Chionomys gud	Kabardino-Balkar Rep., Russia	1	50		100
Microtus longicaudus	New Mexico, USA	1		41	95
M. montanus	New Mexico, USA	1		43	88
M. pennsylvanicus	Wisconsin, USA	3	50		96
M. ochrogaster	Illinois, USA	4		59	44
M. mexicanus	New Mexico, USA	1		46	0
M. juldaschi	Sughd Province, Tajikistan	2	100	100	0
M. gregalis	Novosibirsk Distr., Russia	4	97	62	0
M. brandtii	Chita Distr., Russia	2	100	100	0
M. mandarinus	Buryat Rep., Russia	2	100	100	70
F1 *M. mujanensis x M. maximowiczii*	Buryat Rep., Russia	2	100	100	86

the cytochrome b DNA sequence (Bannikova et al. 2009). Our sample also included a specimen of *Chionomys gud* Satunin, 1909. One species of this genus, *Ch. nivalis*, has previously been shown to possess synaptic X and Y chromosomes (Megias-Nogales et al. 2003). Two representatives of the tribe *Lagurini—Eolagurus luteus* Eversmann, 1840, and *Lagurus lagurus* Pallas, 1773—were used in our study as outgroup species. In addition, F1 hybrids were generated from a cross between two synaptic species, *Microtus mujanensis* Orlov et Kovalskaya, 1978, and *Microtus maximowiczii* Schrenk, 1859. Handling and euthanasia of animals were performed according to protocols approved by the animal care and use committees at the University of Wisconsin, the Zoological Institute of the Russian Academy of Sciences, the Institute of Cytology and Genetics of the Russian Academy of Sciences, and the University of Nagoya.

Spermatocyte spreads were prepared from fresh testis tissue using the drying-down technique (Peters et al. 1997). The cell spreads for imaging by electron microscopy (EM) were stained with silver nitrate (Howell and Black 1980) and covered with a plastic film. After light microscopic examination, cells were transferred to specimen grids, examined, and photographed with an electron microscope (JEM-100, JEOL, Japan) at 80 kV.

Cell spreads for imaging by fluorescent microscopy (FM) were incubated with rabbit polyclonal antibody to human SC lateral element protein SCP3 (1:500, Abcam, Cambridge) and mouse monoclonal antibody to human mismatch repair protein MLH1 (1:30, Abcam), rinsed, and fluorescently labeled with goat anti-rabbit Cy3-conjugated antibodies (1:500, Jackson, West Grove) and goat anti-mouse FITC-conjugated antibodies (1:30, Jackson). Preparations were photographed using an Axioplan 2 Imaging microscope (Carl Zeiss, Germany) equipped with a CCD camera (CV M300, JAI Corporation, Japan), CHROMA filter sets, and ISIS4 image processing package (MetaSystems GmbH, Germany).

Only cells containing complete sets of chromosomes were analyzed. Cells were classified as early, middle, or late pachytene according to the criteria suggested for surface-spread spermatocyte SC preparations of laboratory mice (Moses 1980). Synapsis of the X and Y chromosomes was defined by the formation of a clear synaptonemal complex between the chromosome axes. Accidental associations and crossing of the axes were classified as asynaptic configurations. For cells with synaptic sex chromosomes, we scored the presence or absence of recombination in the pairing regions using immunolocalization patterns of MLH1, a mismatch repair protein of mature recombination nodules (Anderson et al. 1999; Baker et al. 1996; Froenicke et al. 2002). MLH1 signals were only scored if they co-localized with the SC between synapsed sex chromosomes.

Results

Table 1 shows the frequency of pachytene cells containing synapsed XY sex chromosomes in the species studied. No cells with synapsis of the X and Y axes were found by EM analysis for four species: *M. mexicanus*, *M. juldaschi*, *M. gregalis*, and *M. brandtii*. In these species, the axes of the sex chromosomes came together at early pachytene (Fig. 2a) and remained in close proximity through mid- and late pachytene (Fig. 2b, c), often lingering near the periphery of the nucleus and commonly surrounded by electron-dense material. Even though the X and Y axes often overlapped, they never fully aligned with each other to form a true SC. The same chromosome behavior has been described in all other asynaptic species of the genus *Microtus* (Ashley et al. 1989; Borodin et al. 1995; Carnero et al. 1991; Jimenez et al. 1991).

No MLH1 foci were observed on sex chromosomes examined using FM from *M. juldaschi*, *M. gregalis*, or *M. brandtii* despite the presence of clear foci on all autosomal bivalents (Fig. 3a). This indicates an absence of recombination between the X and Y chromosomes.

EM examination of silver-stained spermatocyte spreads from *M. longicaudus*, *M. montanus*, *M. ochrogaster*, and *M. mandarinus* and FM analysis of immunostained SCs of *E. luteus*, *L. lagurus*, *Ch. gud*, *M. pennsylvanicus*, and interspecies hybrids between *M. mujanensis* and *M. maximowiczii* revealed clear pairing between the X and Y chromosomes in most but not all cells (Table 1). Synapsis typically occurred at early pachytene. Terminal parts of the axial elements of the X and Y formed a synaptonemal complex similar in its appearance to that observed in autosomal bivalents (Fig. 2d). At mid-pachytene, the length of the X–Y SC decreased, but the axes of the sex chromosomes remained attached to each other until the end of pachytene (Fig. 2e, f). We detected a single MLH1 focus in the X–Y pairing region along SCs of synaptic species and the interspecies hybrids (Fig. 3b–d). This focus appeared at early pachytene and disappeared during

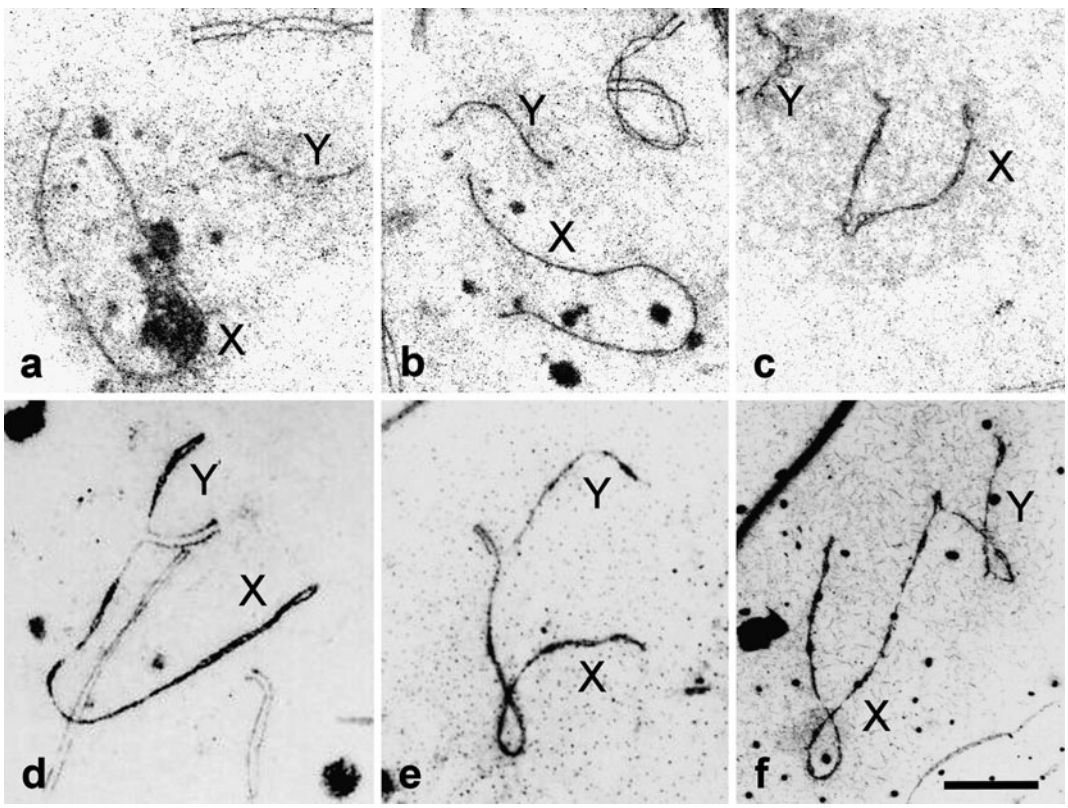

Fig. 2 Electron microphotographs of silver-stained axial elements of X and Y chromosomes in pachytene spermatocytes of asynaptic species *M. juldaschi* (**a**–**c**) and synaptic species *M. montanus* (**d**–**f**). **a**, **d** Early pachytene; **b**, **e** middle pachytene; **c**, **f** late pachytene. *Scale bar*, 2 μm

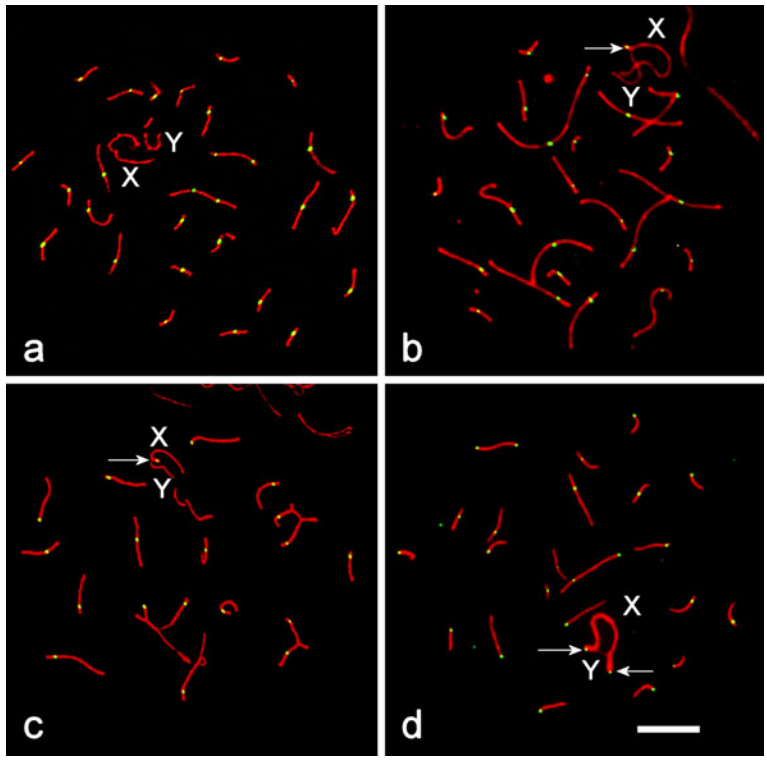

Fig. 3 Immunofluorescent microphotographs of pachytene spermatocytes from *M. juldaschi* (**a**), *M. pennsylvanicus* (**b**), the F_1 hybrid between *M. mujanensis* and *M. maximowiczii* (**c**), and *M. mandarinus* (**d**). *Red*, SCP3; *green*, MLH1. *Arrows* indicate MLH1 foci at XY pairing regions. *Scale bar*, 5 μm

mid-pachytene. In contrast, MLH1 foci on the autosomes were present until late pachytene. This pattern of sex chromosome pairing and recombination has been observed in all synaptic species of rodents studied so far (Anderson et al. 1999; Ashley and Fredga 1994; Borodin et al. 2011, 1997, 1995; Iwasa et al. 1999; Megias-Nogales et al. 2003; Mekada et al. 2001b, 2002).

M. mandarinus was the most interesting species in terms of the synaptic behavior of its sex chromosomes. The specimens we examined had metacentric X chromosomes and telocentric Y chromosomes. We detected about 30% cells with unpaired sex chromosomes. In these cells, the X and Y chromosome axes sometimes folded back, mimicking the appearance of normally paired autosomal bivalents (Fig. 4a). In most cells (about 70%), we observed synapsis between the proximal part of the Y chromosome and distal part of Xp (Fig. 4c). However, in some cells, the Y chromosome was paired along its full length (Fig. 4b). This extensive and apparently non-homologous synapsis between the X and Y chromosomes has been described in *M. mandarinus* (Gu et al. 1999) and in some other species of mammals (Basheva et al. 2008; Borodin 1991; Pack et al. 1993). In about 20% of cells, we observed a circular XY bivalent with two distinct pairing regions of approximately the same length. One involved the proximal part of the Y and distal part of Xp; another consisted of the distal parts of Y and Xq (Fig. 4d). Such circular XY bivalents are observed in human spermatocytes (Speed and Chandley 1990), but, as far as we know, have not been described in the voles.

The XY bivalents of *M. mandarinus* usually contained a single MLH1 focus located very close to the Y centromere. Surprisingly, the second pairing region often contained its own MLH1 focus (Fig. 3d).

Discussion

To identify the lineages along which the asynaptic state arose, we plotted all known synaptic and asynaptic species on an informal supertree of the arvicolines (Fig. 1).

All arvicoline species beyond the genus *Microtus* sensu lato studied so far belonged to the synaptic group. The three additional species studied here—*E. luteus*, *L. lagurus*, and *Ch. gud*—all exhibited synaptic sex chromosome conformations at pachytene. These

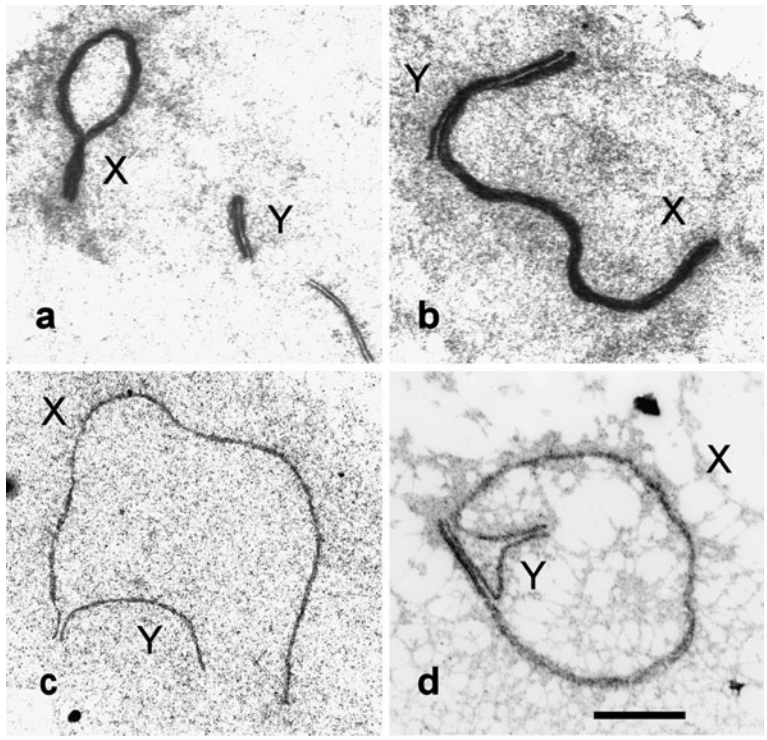

Fig. 4 Synaptic configurations of X and Y chromosomes in pachytene spermatocytes of *M. mandarinus*. **a** Asynapsis. **b** Extended synapsis. **c** Homologous synapsis in one pairing region. **d** Homologous synapsis in two pairing regions. *Scale bar*, 2 μm

results corroborate the prior finding of XY synapsis in *Ch. nivalis* (Megias-Nogales et al. 2003) and strongly suggest that XY pairing at meiosis is the ancestral condition in Microtine voles.

Our analysis uncovers evidence for the loss of this ancestral condition along several independent Microtine lineages.

M. juldaschi, a species recently assigned to the subgenus *Blanfordimys* (Bannikova et al. 2009), was found to have asynaptic XY chromosomes. Two additional species of this subgenus, *Microtus afghanus* and *Microtus bucharensis*, have previously been shown to lack XY synapsis (Borodin et al. 1995). This Central Asian subgenus shows some affinity to the Palearctic phylogenetic lineage of the genus *Microtus* (Bannikova et al. 2010). To date, all Palearctic species examined have asynaptic sex chromosomes, suggesting that XY synapsis was lost along this lineage. However, support for the monophyletic group *Blanfordimys*+Palearctic phylogenetic lineage is weak. Therefore, we cannot rule out the possibility that the loss of XY synapsis occurred in these groups independently.

The monophyly of Nearctic voles is well supported by molecular genetic data (Conroy and Cook 2000; Jaarola et al. 2004). They are considered as a sister group to the Palearctic subgenera *Microtus*, *Agricola*, *Blanfordimys*, *Sumeriomys*, and *Terricola* (all asynaptic) and the Asian subgenera *Pallasiinus* and *Alexandromys* (all synaptic). We found synaptic sex chromosomes in four Nearctic species (*M. longicaudus*, *M. montanus*, *M. pennsylvanicus*, and *M. ochrogaster*), whereas one species (*M. mexicanus*) was asynaptic. The observed heterogeneity of X–Y pairing patterns within the Nearctic voles is consistent with another independent loss of XY synapsis.

Variability in XY pairing patterns was also detected in the lineage including *M. (Stenocranius) gregalis*, *M. (Lasiopodomys) brandtii*, and *M. (Lasiopodomys) mandarinus.* These three species belong to the earliest and longest branch sister to all other lineages of the genus *Microtus* sensu lato (Abramson et al. 2009; Bannikova et al. 2009; Jaarola et al. 2004). *M. gregalis* and *M. brandtii* are both asynaptic, suggesting that XY asynapsis also evolved along this branch, independently from the other asynaptic branches described above.

In contrast, the sex chromosomes of *M. mandarinus* pair and recombine at pachytene. However, several observations favor the argument that XY synapsis in *M. mandarinus* represents a derived condition. First, two other species belonging to the same lineage are

asynaptic (*M. gregalis* and *M. brandtii*). Second, the pattern of XY pairing observed in this species is pronouncedly different from that observed in all other arvicolines. The XY pair of this species contains two pairing regions. Both are relatively long and both are involved in regular recombination, similar to the pseudoautosomal pairing regions in human. These regions may have originated by de novo translocations of autosomal material, as suggested for the human pairing regions (Graves et al. 1998).

Our results indicate that the ability of the sex chromosomes to pair and recombine in male meiosis has been lost independently at least three times during the evolution of Microtine rodents: (1) in the lineage including subgenera *Stenocranius* and *Lasiopodomys*; (2) in the Nearctic species *M. (Aulacomys) mexicanus*; and (3) in Palearctic phylogenetic lineage including subgenera *Microtus*, *Sumeriomys*, *Terricola*, *Agricola*, *Blanfordimys*, and *Iberomys*. This may be an underestimate as the latter three subgenera occupy basal positions in the Microtine tree and their relation to the Palearctic lineage remains controversial (Bannikova et al. 2010; Jaarola et al. 2004). They may have acquired the asynaptic condition independently.

We speculate that these losses were facilitated by the emergence of an as yet uncharacterized cellular mechanism for sequestering the sex chromosomes at meiosis in the common ancestor of *Microtini*. It has been suggested that some components of the axial elements of SC may participate in the segregation of sex chromosomes in asynaptic species of mammals (de la Fuente et al. 2007; Page et al. 2003). The evolution of a novel mechanism for ensuring proper sex chromosome segregation without imposing a requirement for pairing and recombination would reduce (or eliminate) selective constraint on XY synapsis, setting the stage for subsequent losses of XY pairing in multiple lineages. XY pairing has been completely lost in asynaptic species. Interestingly, in some synaptic species, we observe a high frequency of pachytene cells with X–Y asynapsis: up to one third in *M. mandarinus* and two thirds in *M. ochrogaster*.

We do not know whether losses of XY pairing have been caused by deletions, rearrangements of the ancestral pairing region, or by epigenetic modification. Acosta et al. (2011) found no correlation between synaptic and asynaptic classes of sex chromosomes and the presence or absence of sequence homology in their euchromatic regions. Thus, the existence of sequence homology between the X and Y is not required for sex chromosome pairing in male meiosis (Acosta et al. 2011). However, data on MLH1 immunolocalization at the XY bivalent of the synaptic species such as in *M. maximowiczii*, *M. mujanensis*, and *M. fortis* described earlier (Borodin et al. 2011) and in *M. mandarinus* and *M. pennsylvanicus* described here indicate that homologous recombination does occur in the pairing region. This is clear evidence that the synaptic species of the genus *Microtus* retain sequence homology between the X and Y.

Interestingly, this sequence homology remains unaltered even after speciation events. *M. mujanensis* ($2n=38$, FN=46) and *M. maximowiczii* ($2n=40$, FN=54), two synaptic species, diverged from each other ~0.3 Mya. Although their genomes are distinguished by several chromosomal rearrangements, the X chromosome of *M. mujanensis* pairs normally with the Y chromosome of *M. maximowiczii* during the meiotic prophase in interspecies hybrids. Future work comparing the molecular structure of pairing regions in synaptic species versus the orthologous regions in asynaptic species could help illuminate the genetic mechanisms of XY synaptic gain and loss across the vole phylogeny.

Acknowledgments This work was supported by research grants from Russian Foundation for Basic Research (to PMB, EAB, AAT, OAD, FNG, IVK), Siberian and Far Eastern Departments of Russian Academy of Sciences (to PMB and IVK), National Science Foundation Predoctoral Fellowship (to BLD), and Japan Science Society Sasagawa Scientific Research Grant (to KM). We thank Mrs. Marina Rodionova for her help in SC spreading; Drs. Andrei Dobrotvorsky, Victor Panov, Yuichi Narita, Bruce Cushing, Nancy Cushing, and Ms. Roni Yi-Ju Chen for providing of animals; Drs. Sen-ichi Oda, Kazuhiro Koyasu, Masashi Harada, and Vladimir Lebedev for valuable advices; and the Microscopic Center of the Siberian Department of Russian Academy of Sciences for granting access to microscopic equipment.

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