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A- and B-chromosome pairing and recombination in male meiosis of the silver fox (*Vulpes vulpes* L., 1758, Carnivora, Canidae)

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Abstract We examined A- and B-chromosome pairing and recombination in 12 males from the farm-bred population of the silver fox (2*n*=34+0–10 Bs) by means of electron and immunofluorescent microscopy. To detect recombination at A and B chromosomes, we used immunolocalisation of MLH1, a mismatch repair protein of mature recombination nodules, at synaptonemal complexes. The mean total number of MLH1 foci at A-autosomes was 29.6 foci per cell. The XY bivalent had one MLH1 focus at the pairing region. Total recombination length of the male fox genome map was estimated as 1,530 centimorgans. We detected single MLH1 foci at 61% of linear synaptic configurations involving B chromosomes. The distribution of the foci along B- and A-bivalents was the same. This may be considered as a first molecular evidence that meiotic recombination does occur in mammalian B chromosomes. There was no correlation between the number of synaptic configurations involving B chromosomes per cell and the recombination rate of the A-genome.

Keywords B chromosomes · Meiotic recombination · Synaptonemal complex · MLH1 · Fox · *Vulpes vulpes*

Abbreviations

ACA	Anti-centromere antibody
BSA	Bovine serum albumin
cM	Centimorgan
DAPI	4′-6-diamidino-2-phenylindole
Cy3	Orange fluorescing cyanine
FITC	Fluorescein isothiocyanate
MLH1	Homolog of prokaryotic mutL 1 mismatch repair protein
PBS	Phosphate buffered saline
SC	Synaptonemal complex
SCP3	Synaptonemal complex protein 3
S.D.	Standard deviation

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Introduction

B chromosomes are accessory genomic elements to the so-called A or standard genome. They have been found in many species of plants and animals. Populations usually show a polymorphism for the number and morphology of B chromosomes (Camacho et al. 2000; Jones and Rees 1982).

Typically, B chromosomes are transmitted in a non-Mendelian fashion due to their unstable mitotic and/or meiotic behavior (Bidau et al. 2004; Camacho et al. 2000; Jimenez et al. 2000; Jones 1991, 1995; Perfectti et al. 2004; Santos et al. 1993). For this reason, B chromosomes are considered as selfish genetic elements or genomic parasites engaged in a continuous conflict with the A-genome (Bell and Burt 1990; Camacho et al. 2000, 2002).

One of the most common and less understood effects of B chromosomes on the A-genome is that their presence modifies the rate of meiotic intra-chromosomal recombination (crossing over) of the carrier cell. B chromosomes have been shown to affect chiasma frequency and/or distribution, and between-cell variance (Brandham and Bhattarai 1977; Jones and Rees 1967; Jones 1991, 1995; Rees and Dale 1974; Ward 1976). Different number of B chromosomes may affect the host recombination in a zig-zag pattern (the odd–even effect) (Camacho et al. 2004).

Meiotic behavior of B chromosomes has been extensively studied in plants and insects (Jones and Rees 1982), but studies in mammals are scarce. As far as we know, the meiotic pattern of B chromosomes has been studied in only four species: Korean field mouse *Apodemus peninsulae* (Kolomiets et al. 1988), yellow-necked mouse *Apodemus flavicollis* (Banaszek and Jadwiszczak 2006), raccoon dog *Nyctereutes procyonoides* (Shi et al. 1988) and silver fox *Vulpes vulpes* (Radzhabli et al. 1978; Switonsky et al. 1987).

The silver fox *V. vulpes* ($2n$=34+0–10 Bs) is an especially interesting model for the analysis of meiotic behavior of B chromosomes for the following reasons. B chromosomes are present in wild and in farm-bred populations of this species. They are mitotically unstable and different cells of the same individual may contain different number of B chromosomes (Beliaev et al. 1974a, b; Radzhabli et al. 1978). They occur in two canid species of different genera (*V. vulpes* and *N. procyonoides*) and in both species they carry functional copies of the c-kit proto-oncogene (Graphodatsky et al. 2005; Yudkin et al. 2007).

In this study, we examined synapsis and recombination of B chromosomes in the farm-bred silver foxes by means of electron and immunofluorescent microscopy. To detect recombination at A and B chromosomes we used immunolocalization of MLH1, a mismatch repair protein of mature recombination nodules, at synaptonemal complexes (SC). This approach has proved to give reliable estimates of total recombination rate, as well as the frequency and distribution of recombination events in individual chromosomes of several species of mammals (Anderson et al. 1999; Basheva et al. 2008; Borodin et al. 2007, 2008, 2009; Codina-Pascual et al. 2006; Froenicke et al. 2002; Hassold et al. 2004; Lynn et al. 2002; Sun et al. 2006).

Materials and methods

Spermatocyte spreads were prepared using the drying-down technique (Peters et al. 1997). Testes were isolated 1 month before the breeding season (December 2007) from twelve 9-month-old male foxes maintained at the Experimental farm of the Institute of Cytology and Genetics.

For electron microscopic examination the spreads were stained with silver nitrate (Howell and Black 1980) and covered with plastic film. The spreads after light-microscopic examination were transferred to specimen grids and examined with electron microscope JEM-100 (JEOL, Japan) at 80 kV.

The immunostaining protocol was performed according to Anderson et al. (1999). The slides were incubated overnight at 37°C with a rabbit polyclonal antibody against human SC lateral element protein SCP3 (Abcam, Cambridge) diluted to a concentration of 1:500, a mouse monoclonal antibody to human mismatch repair protein MLH1 (1:50, Abcam, Cambridge), and a human anti-centromere antibody (ACA; 1:100, Antibodies Inc., Davis) in 3% bovine serum albumin in phosphate buffered saline (PBS). Slides were washed in 1× PBS and incubated for 40 min at 37°C with goat anti-rabbit Cy3-conjugated antibodies (1:500, Jackson, West Grove), goat anti-mouse FITC-conjugated antibodies (1:50, Jackson), and donkey anti-human FITC-conjugated antibodies (1:100, Vector Laboratories). Slides were washed with PBS, rinsed briefly with distilled water, dried, and mounted in Vectashield with DAPI (Vector Laboratories) to stain DNA and reduce fluorescence fading.

The preparations were analyzed with 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). Brightness and contrast of the images were enhanced using PaintShopPro 7.0.

MLH1 foci were counted in 427 pachytene cells containing complete sets of completely paired A-bivalents (Fig. 1). The centromere position for each SC in the pachytene cells was identified by an ACA focus. Although we used the same fluorochrome for detection of the ACA and MLH1 antibodies, ACA foci differed from MLH1 foci by their brighter and more diffuse staining (Fig. 1). MLH1 signals were only scored if they were localized on a SC. The length of the SC of each bivalent was measured in micrometers using MicroMeasure 3.3 (Reeves 2001).

Results

Chromosome pairing

Figure 1 shows a spermatocyte spread of the male silver fox (2*n*=34+4 Bs) immunolabeled with antibodies to SCP3, MLH1, and centromere proteins. A mean (±SD) total length of A-autosomal SC was 210.7±21.22 μm. B chromosomes were clearly distinguishable from the A chromosomes by their morphology and size. All A chromosomes were biarmed, while all B chromosomes were acrocentric. Average (±SD) length of B-chromosome SC (1.68±0.5 μm) was about six times smaller than the average length of the smallest A-bivalent (10.6±0.5 μm).

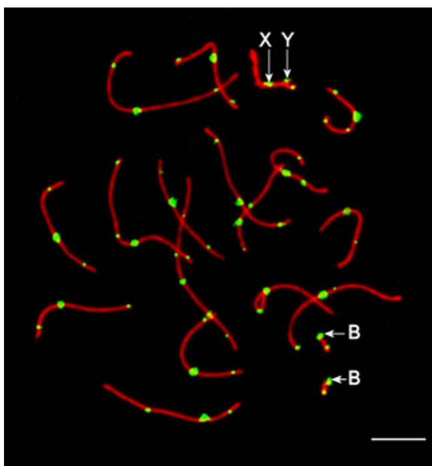

Fig. 1 Spread from a fox spermatocyte at pachytene immunolabeled with antibodies to SCP3 (*red*), MLH1 (*green*), and centromere proteins (*green*). Centromeres differ from MLH1 foci by their brighter and more diffuse staining. *Arrows* indicate centromeres of *X*, *Y*, and B chromosomes. Bar=5 μm

Under the electron microscope we observed various synaptic configurations of B chromosomes: bivalents, univalents and multivalents (Fig. 2). Most of the bivalents were completely paired (Fig. 2a). Rarely we observed incompletely paired bivalents (Fig. 2b) and bivalents containing partners of different sizes (Fig. 2c). A majority of univalents appeared in a "hairpin" configuration (Fig. 2d). Multivalent configurations were very rare and always showed extensive asynapsis (Fig. 2e). B chromosomes were often but not always found in a close proximity to the XY bivalent. They never formed proper SC with the sex chromosomes or any of the autosomes, although in one cell we detected a close alignment between B- and A-bivalents (Fig. 2f).

Immunofluorescent microscopic analysis of 427 pachytene cells revealed 793 synaptic configurations involving B chromosomes. There were 20 trivalents among them and 773 linear synaptic configurations. Resolution of fluorescent microscopy did not permit an unambiguous discrimination between bivalents and univalents of B chromosomes.

A variation in the number of synaptic configurations involving B chromosomes between the individuals and between the cells within the individuals is shown in the Table 1. Only one specimen (No. 8) had the same number of the linear synaptic configurations (one) in all cells examined. In other specimens the number of synaptic configurations of B chromosomes varied between the cells. There usually was a dominant clone and a series of minor clones containing smaller and larger number of B chromosomes. The highest level of mosaicism was observed in the male No 6. This indicates that mosaicism for B chromosomes, described in somatic cells of the silver fox (Beliaev et al. 1974b), is also present in germ line cells.

Recombination

The mean (±SD) number of MLH1 foci at A-autosomes was 29.6±2.4 foci per cell. We detected a single MLH1 focus in the X–Y pairing region in all cells examined. It was usually located very close to the Xp–Yq telomeres (Fig. 1). To estimate in centimorgans (cM) the recombination length of the male fox genome, we multiplied the average number

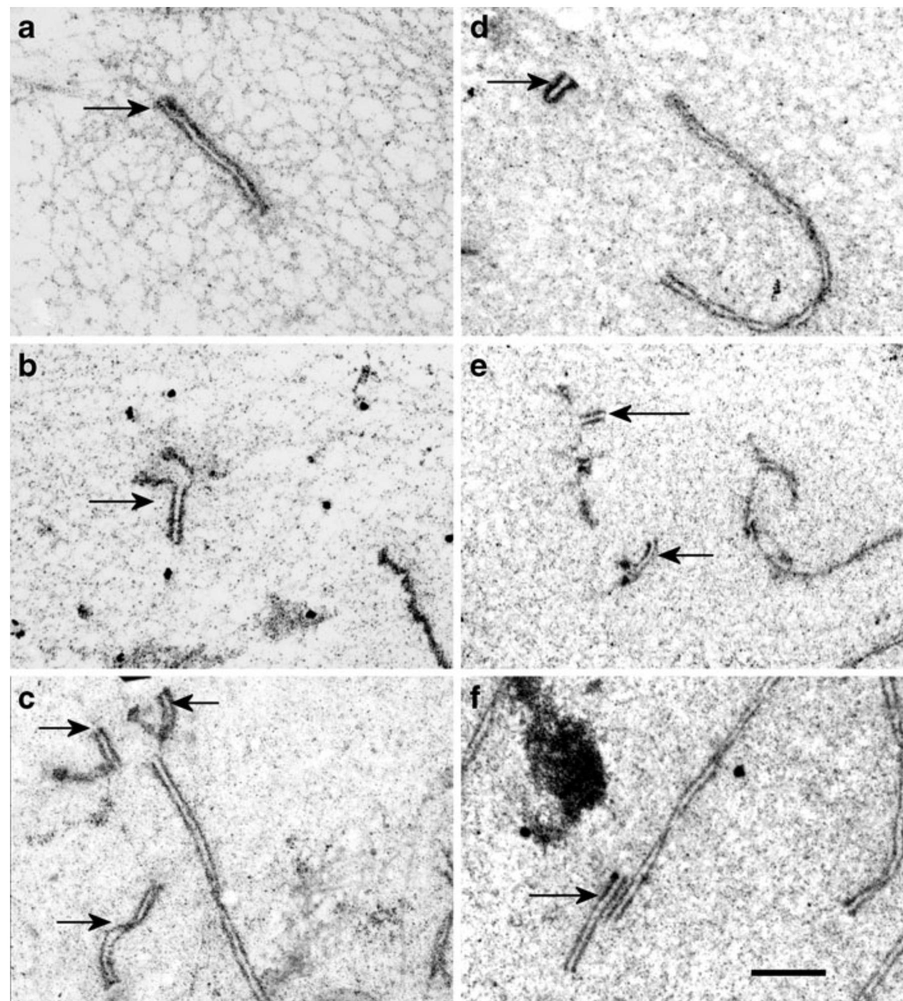

Fig. 2 Synaptic configurations of B chromosomes: silver staining. Arrows indicate configurations involving B chromosomes: **a** completely paired bivalent, **b** incompletely paired bivalent, **c** two bivalents containing partners of different sizes (*top*) and completely paired bivalent (*below*), **d** hairpin-like univalent, **e** incompletely paired trivalent (*top*) and completely paired bivalent (*below*), **f** closely aligned A- and B-bivalents. Bar: 1 μm

of MLH1 foci per cell by 50 map units (one recombination event=50 cM), which gave 1,530 cM. This is very close to the estimate of the length of sex-averaged genetic map of the silver fox coming from meiotic linkage analysis: 1,480.2 cM (Kukekova et al. 2007).

Figure 3 shows the distribution of MLH1 foci along bivalents grouped by their size rank. In all A-bivalents we observed prominent peaks of MLH1 foci near the distal ends and a paucity of them near the centromeres. This pattern is common to all mammals studied so far (Anderson et al. 1999; Basheva et al. 2008; Borodin et al. 2007, 2008, 2009; Froenicke et al. 2002; Sun et al. 2006).

We detected single MLH1 foci at 61% of linear synaptic configurations involving B chromosomes (Fig. 1). The mean (±SD) length of their SC was 1.67±0.47 μm. It did not differ from the length of those without MLH1 foci (1.68±0.48 μm). Thus, an occurrence of MLH1 foci at the linear synaptic configurations involving B chromosomes did not depend on their size. As we mentioned above, the resolution of fluorescent microscopy did not permit a reliable discrimination between bivalents and univalents of B chromosomes. We might presume, however, that all synaptic configurations containing MLH1 foci were B-bivalents. Although, probably not all B-bivalents contained MLH1 foci. The data on male No. 8 might give an estimate of MLH1 focus occurrence at B-bivalents. All pachytene cells of this male contained one linear B-SC, which most probably was B-bivalent. MLH1 foci were observed at 87% of them.

The distribution of the MLH1 foci along the B-bivalents followed the pattern typical to A–SC: a high

Table 1 Number of pachytene cells containing various numbers of synaptic configurations of B chromosomes

Fox ID	No. of cells containing						
	Linear synaptic configurations						Trivalents
	0	1	2	3	4	5	1
1	8	31	5				
2		3	8	2			1
3		41	7	1			
4		2	31	3			1
5		37	4				
6			6	14	10	10	7
7		1	26	7	1		
8		31					
9	3	12	18				5
10	1	1	23	1			1
11	1	4	17	4			1
12	1	2	24	24	2		4
Total	14	165	169	56	13	10	20

frequency near the distal end and gradual decrease toward the centromere (Fig. 3). Most trivalents (19 out of 20 observed) had single MLH1 focus which was usually located at the point of switching of pairing partners.

We did not find a significant correlation between the number of synaptic configurations involving B chromosomes per cell and number of MLH1 foci at A-autosomes (r=0.01, p>0.05).

Discussion

The pattern of B-chromosome pairing found in this study was similar to that described earlier in the fox (Switonsky et al. 1987) and other mammals: raccoon dog (Shi et al. 1988) and Asian field mouse (Kolomiets et al. 1988). In all three species supernumerary chromosomes showed mitotic instability and mosaicism for their number in the germ line. Electron microscopy of surface spread SCs revealed that B chromosomes of these species were able to pair homologously and to form various synaptic configurations (univalents, bivalents and multivalents) depending on the number of B chromosomes in the cell.

In this study, we detected a regular presence of MLH1 foci at B-bivalents and trivalents. The distribution of the foci along B- and A-bivalents was the same. This can be considered as the first molecular evidence that crossingover does occur at mammalian B chromosomes. Radzhabli et al. (1978) and Switonsky et al. (1987) observed bivalents of B chromosomes at diakinesis—metaphase I in the silver fox; however, no chiasmata were seen. Small size and heterochromatic content make very difficult the detection of chiasmata between B chromosomes using classic light-microscopic cytogenetic techniques. Moreover, in this study we found that most crossovers at the fox B chromosomes were predominantly located near their distal ends. The distally located chiasmata are very difficult to detect even at A chromosomes.

In few cases, true chiasmata were observed in insects and plants. Chiasmata between the euchromatic portions of B chromosomes were reported in two species of grasshoppers *Dichroplus pratensis* (Bidau 1987) and *Metaleptea brevicornis* (Grieco and Bidau 2000). In plants, chiasmata were observed in B chromosomes of rye (Jones and Rees 1967). It has been suggested that specific genes that control meiotic transmission of B chromosomes in rye were located at the sites of chiasma formation (Jimenez et al. 2000).

Synapsis and recombination between the fox B chromosomes were apparently facilitated by their homology to each other. Using FISH, it has been shown that a whole chromosome DNA probe derived from a single B chromosome will label all other B chromosomes as well as centromeric regions and interstitial heterochromatin blocks of most A chromosomes (Yang et al. 1999). This indicates that the fox B chromosomes contain A-derived repeated sequences as observed in B chromosomes of other mammalian species (Karamysheva et al. 2002; Matsubara et al. 2008; Rubtsov et al. 2004; Tanic et al. 2000; Trifonov et al. 2002).

Regular recombination between the fox B chromosomes may lead to homogenization of their genetic content. On the other hand unequal recombination of repeated sequences may generate a variation between B chromosomes in their size.

Although B chromosomes are heterochromatic, they contain copies of the proto-oncogene c-kit. (Graphodatsky et al. 2005). It encodes a transmem-

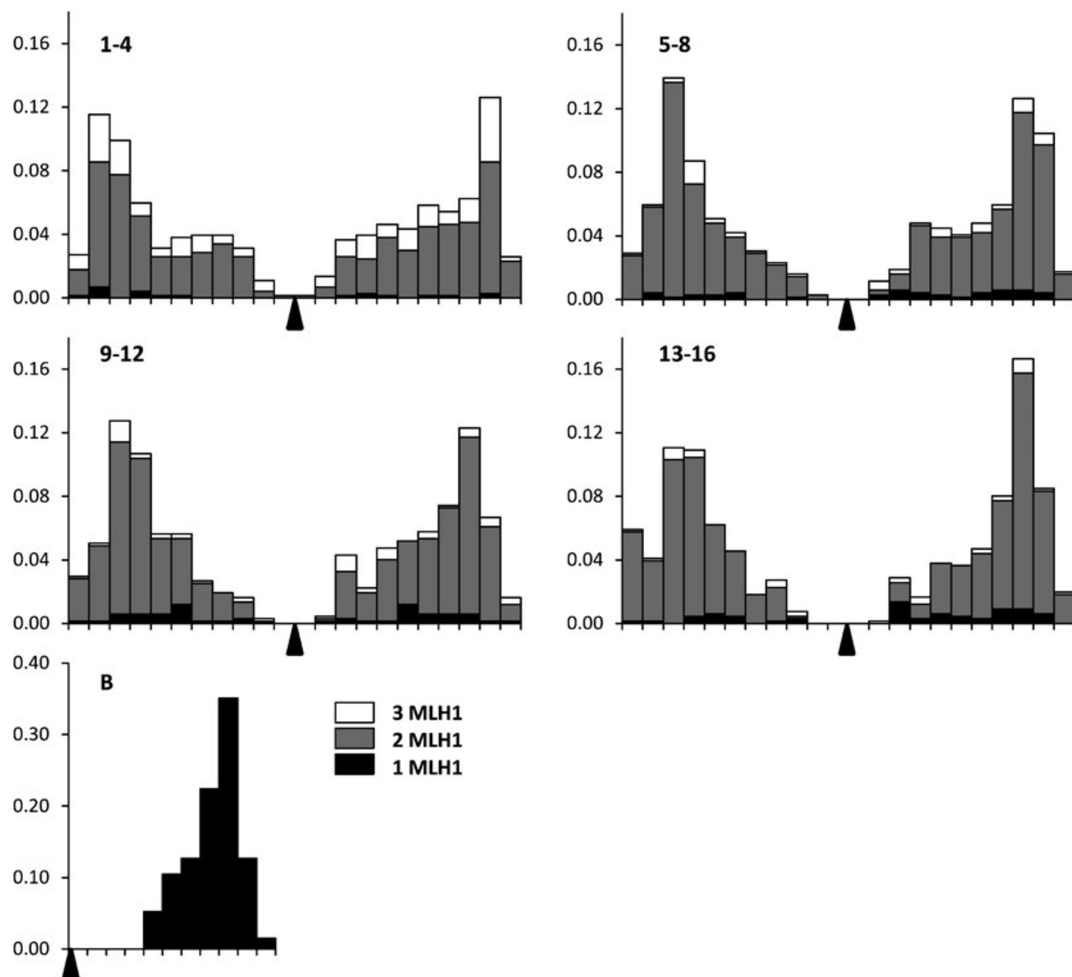

Fig. 3 Distribution of MLH1 foci along bivalents grouped by their size rank. The SCs size ranks included in each group are indicated in *top left corners* of the graphs. The *x*-axis shows the position of MLH1 foci in relation to centromere and telomere, the marks on this axis are separated by 0.1 of the SC arm length. The *y*-axis indicates the frequency of MLH1 foci in each interval

brane tyrosine kinase which plays an important role in proliferation and migration of melanoblasts, hematopoietic progenitors and primordial germ cells. The copies of this gene located in the fox B chromosomes are apparently intact because their exons show a very small difference with active autosomal canine c-kit (Yudkin et al. 2007). This may indicate that these copies retain the original function and their integrity has been preserved by natural selection. Recombination of B chromosomes detected in this study may be considered as a mechanism protecting c-kit copies from mutational meltdown and degeneration (Rice 1994).

Probable acquisition of c-kit, a gene important for host development, by the fox B chromosomes may be viewed as a sign of co-adaptation between A- and B-genomes. The same gene was found in B chromosomes of other canid species, the raccoon dog *N. procyonoides* (Yudkin et al. 2007). This may indicate that the fox and raccoon dog B chromosomes might have been inherited from a common ancestor (ca 12.5 MYA). Such a long history of co-evolution between fox A- and B-genomes should have led to a resolution of their genomic conflict and a reduction of parasitic properties of B chromosomes.

Analysis of evolutionary dynamics of parasitic B chromosomes in insects indicates that their rapid invasions due to non-Mendelian transmission are usually followed by a reduction and elimination of B-chromosome drive due to selection of the drive

suppressors located at A chromosomes (Camacho et al. 1997). While the invasion may occur within several dozens of generations, the neutralization of B chromosome takes much longer time and its success depends on the genetic variation available, which in turn depends on the recombination rate of A-genome. An increased recombination of A chromosomes observed in B-chromosome carriers in many plant and animal species is considered as a sign of genomic conflict, as an adaptive response in the host provoked by the parasitic B chromosome (Bell and Burt 1990; Camacho et al. 2000, 2002; Jones 1995). In the silver fox, we did not found an effect of B chromosomes on A-genome recombination. This indicates that the fox B chromosomes lost their parasitic properties.

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