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Chromosome synapsis, recombination and epigenetic modification in rams heterozygous for metacentric chromosome 3 of the domestic sheep *Ovis aries* and acrocentric homologs of the argali *Ovis ammon*

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Hybridization of domestic animal breeds with their wild relatives is a promising method for increasing the genetic diversity of farm animals. Resource populations derived from the hybridization of various breeds of domestic sheep with mouflon and argali are an important source of breeding material. The karyotypes of argali and domestic sheep differ for a Robertsonian translocation, which occurred in the common ancestor of mouflon and domestic sheep (*Ovis aries*) due to the centric fusion of chromosomes 5 and 11 of the argali (*O. ammon*) into chromosome 3 of sheep. It is known that heterozygosity for translocation can lead to synapsis, recombination and chromosome segregation abnormalities in meiosis. Meiosis in the heterozygotes for translocation that distinguishes the karyotypes of sheep and argali has not yet been studied. We examined synapsis, recombination, and epigenetic modification of chromosomes involved in this rearrangement in heterozygous rams using immunolocalization of key proteins of meiosis. In the majority of cells, we observed complete synapsis between the sheep metacentric chromosome and two argali acrocentric chromosomes with the formation of a trivalent. In a small proportion of cells at the early pachytene stage we observed delayed synapsis in pericentromeric regions of the trivalent. Unpaired sites were subjected to epigenetic modification, namely histone H2A.X phosphorylation. However, by the end of the pachytene, these abnormalities had been completely eliminated. Asynapsis was replaced by a nonhomologous synapsis between the centromeric regions of the acrocentric chromosomes. By the end of the pachytene, the γH2A.X signal had been preserved only at the XY bivalent and was absent from the trivalent. The translocation trivalent did not differ from the normal bivalents of metacentric chromosomes for the number and distribution of recombination sites as well as for the degree of centromeric and crossover interference. Thus, we found that heterozygosity for the domestic sheep chromosome 3 and argali chromosomes 5 and 11 does not cause significant alterations in key processes of prophase I meiosis and, therefore, should not lead to a decrease in fertility of the offspring from interspecific sheep hybridization.
Key words: *Ovis aries*; immunostaining; meiosis; synaptonemal complex; recombination; Robertsonian translocation.

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Синапсис, рекомбинация и эпигенетическая модификация хромосом у баранов, гетерозиготных по метацентрической хромосоме 3 домашней овцы *Ovis aries* и акроцентрическим гомологам архара *Ovis ammon*

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Гибридизация пород домашних животных с их дикими сородичами может служить перспективным методом повышения генетического разнообразия сельскохозяйственных животных. Ресурсные популяции, полученные на основе гибридизации различных пород домашних овец с муфлоном и архаром, являются важным источником селекционного материала. Кариотипы архара и домашней овцы различаются по робертсоновской транслокации, возникшей у общего предка муфлона и овец (*Ovis aries*) за счет центрического слияния хромосом 5 и 11 архара (*O. ammon*) с образованием хромосомы 3 овцы. Известно, что гетерозиготность по транслокациям может приводить к нарушениям синапсиса, рекомбинации и сегрегации хромосом в мейозе. Осо-

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бенности протекания мейоза у баранов, гетерозиготных по транслокации, различающей кариотипы овец и архаров, до сих пор не исследованы. Мы изучали синапсис, рекомбинацию и эпигенетическую модификацию хромосом, вовлеченных в данную перестройку у гетерозигот, с использованием иммунолокализации ключевых белков мейоза. В большинстве клеток наблюдался полный синапсис между метацентрической хромосомой овцы и двумя акроцентрическими хромосомами архара с образованием тривалента. В небольшой доле клеток на стадии ранней пахитены наблюдалась задержка синапсиса в перицентромерных районах тривалента. Неспаренные участки подвергались эпигенетической модификации: фосфорилированию гистона H2A.X. Однако к концу пахитены эти нарушения полностью устранялись. Асинапсис замещался негомологичным синапсисом между перицентромерными районами акроцентрических хромосом. К концу пахитены сигнал γH2A.X сохранялся только на половом биваленте и отсутствовал на триваленте. По числу и распределению рекомбинационных сайтов, степени центромерной и кроссоверной интерференции транслокационный тривалент не отличался от нормальных бивалентов метацентрических хромосом. Таким образом, установлено, что гетерозиготность по хромосоме 3 домашней овцы и хромосомам 5 и 11 архара не вызывает существенных изменений в ключевых процессах профазы I мейоза и, следовательно, не должна приводить к снижению плодовитости у потомков от межвидовой гибридизации овец.
Ключевые слова: *Ovis aries*; иммуноокрашивание; мейоз; синаптонемные комплексы; рекомбинация; робертсоновские транслокации.

Introduction

Hybridization of domestic animals with their wild relatives is a promising method for increasing the genetic diversity of farm breeds and introducing factors of resistance to diseases and extreme environmental factors into their genomes (Serebrovsky, 1935). This approach has been used in sheep breeding. Resource populations obtained from hybrids of various breeds of domestic sheep (*Ovis aries*: OAR) with mouflon (*O. orientalis*) and argali (*O. ammon*: OAM) serve as a source of valuable alleles and allelic combinations for subsequent selection (Deniskova et al., 2016).

However, karyotypic differences between domestic and wild species might affect the fertility of the hybrids. The argali karyotype contains two pairs of metacentric chromosomes ($2n = 56$), and the karyotypes of the mouflon and domestic sheep contain three pairs of metacentrics ($2n = 54$). Differences in the diploid number of chromosomes occurred due to the Robertsonian fusion between the chromosomes 5 and 11 argali: rob (OAM5;11) that originated in the common ancestor of mouflon and sheep and resulted in the sheep chromosome 3 (OAR3) (Bunch et al., 1998). Hereinafter, for the argali chromosomes we use the standard nomenclature of the Bovidae chromosomes (Popescu et al., 1996). Theoretically, heterozygosity for Robertsonian translocations should lead to significant disruptions of meiosis and a decrease in fertility. Disturbances of meiosis can occur due to the spatial complexity of the presynaptic alignment and subsequent synapsis between metacentric and acrocentric homologues (Borodin et al., 1998). Delayed synapsis may lead to transcriptional inactivation of unpaired chromatin, apoptosis and death of generative cells (Burgoyne, Mahadevaiah, 1993; Burgoyne et al., 2009). Even in the case of successful synapsis and normal recombination, nondisjunction of chromosomes involved in the trivalent may lead to the formation of unbalanced gametes and a decrease in fertility of heterozygotes (Garagna et al., 2014).

Despite this, Robertsonian translocations are the most common variant of evolutionary chromosomal rearrangements in mammals and the common cause of karyotypic difference between closely related species (Ferguson-Smith, Trifonov, 2007). Polymorphism for Robertsonian translocations is widespread in populations of many species (Dobigny et al., 2017) including breeds of domestic sheep (Broad et al., 1997) because they rarely cause phenotypic effects. In most cases, fertility does not decrease in simple heterozygotes carrying the metacentric chromosome and two homologous acrocentric chromosomes. Moreover, fertility remains normal even in the case of simple heterozygosity for several Robertsonian translocations (Bruère, Ellis, 1979; Dobigny et al., 2017). Reduced fertility is usually found in complex heterozygotes for several Robertsonian translocations involving the same shoulders of chromosomes: hybrids with monobrachial homology (Medarde et al., 2015).

Domestic sheep shows intraspecific polymorphism for five Robertsonian translocations: rob(6;24), rob(9;10), rob(7;25), rob(5;8), and rob(8;22) (Broad et al., 1997). Electron microscopic analysis of synaptonemal complexes (SC) in male heterozygotes for the translocations rob(6;24), rob(9;10) and rob(7;25) revealed a relatively high frequency of cells with delayed synapsis in trivalents formed by metacentric chromosome and two acrocentric homologues. Another abnormality detected was associations between the unpaired pericentromeric areas of the acrocentric elements of trivalent and the XY bivalent (Dai et al., 1994a, b). At the same time, it was shown that simple heterozygotes for one or several of these translocations retain normal fertility, although they have a slightly increased level of chromosome nondisjunction (Bruère, Ellis, 1979).

Meiosis in rams heterozygous for the translocation rob(OAM5;11), have not been studied yet. Analysis of meiosis is important in the light of experiments for creating resource populations based on hybrids of various breeds of domestic sheep with mouflon and argali (Deniskova et al., 2016).

In this study, we investigated synapsis, recombination, and epigenetic modification of chromosomes involved in rob(OAM5;11), using immunolocalization of key meiotic proteins. The lateral elements of the SC were visualized using antibodies to the SYCP3 protein. Epigenetic modifications of the regions containing unrepaired double-strand DNA breaks were detected by antibodies to the phosphorylated form of histone H2A.X (γH2A.X) (Rogakou et al., 1998). We evaluated the number and distribution of recombination nodules using antibodies to MLH1, the mismatch repair protein (Anderson et al., 1999). It is known that the global distribution of recombination events by chromosomes is mainly determined by the

size of the chromosome and the centromeric-telomeric gradient (Kleckner et al., 2003). The genetic content determines the positions of recombination hot spots within 1–2 thousand base pairs and does not make a significant contribution to the chromosome-wide recombination pattern (Lichten, Goldman, 1995). Chromosomes of most of the studied species of vertebrates show the distal peaks of recombination (Ruiz-Herrera et al., 2017). The distribution of crossover sites is also affected by centromeric interference (suppression of recombination near the centromere) and crossover interference (reduction of the probability of a new crossing over to occur near to one that has already arisen) (Zickler, Kleckner, 2015). The greater the distance between adjacent recombination points, the greater the interference. Little is known about the number and distribution of MLH1 sites in domestic and wild sheep species (Muñoz-Fuentes et al., 2015; Ruiz-Herrera et al., 2017). The study of these characteristics in heterozygotes for species-specific evolutionary translocation rob(OAM5;11) is of particular interest.

Materials and methods

Meiotic chromosome spreads were prepared from the testes of mature 6–9 months old rams breed in the experimental farm of the Federal Scientific Center for Animal Husbandry. Rams heterozygous for rob(OAM5;11) were obtained in the crosses shown in Fig. 1. Rams of the Romanov breed were used as representatives of the standard karyotype.

Testes were isolated during castration. The maintenance, anesthesia and castration were carried out in accordance with the international and national rules of humane treatment of animals according to the protocol approved by the Commission on Bioethics of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (protocol No. 35 of October 16, 2016).

SC spreads were prepared by the method of Peters et al. (1997) with modifications. The testes were removed from *tunica albuginea* and a small fragment of the testis about 0.5 cm^3 in size was placed in a hypotonic extraction buffer (30 mM Tris, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, pH 8.2) for 90–110 minutes. Then the seminiferous tubules were macerated in 40 µl of a 0.1 M sucrose solution at pH 8.2. The debris was removed and a suspension of testicular cells was re-suspended several times. One drop (20 µl) of the resulted suspension was placed onto slides moistened with 1 % paraformaldehyde solution and slowly distributed over the surface by tilting the slide. The slides were left to dry in a humid chamber for 2 hours. The preparations were washed in 0.4 % Kodak PhotoFlo, dried and stored until staining in sealed containers at –20 °C.

Immunostaining of the SC spread was performed according to the method of Anderson et al. (1999) with modifications. A solution of 400 µl of 10 % PBT (PBS (phosphate-buffered saline), 0.05 % Tween-20, 3 % BSA (Sigma-Aldrich, USA)) was applied to the slides at room temperature for 45 minutes to block non-specific antibody binding. Then the preparations were incubated for 12 hours at 37 °C with primary antibodies. Depending on the task, the following antibodies were used: rabbit polyclonal antibodies to human SYCP3 protein (Abcam, UK) at a dilution of 1 : 500; mouse monoclonal antibodies to human MLH1 protein (Pharmingen, USA) at a dilution of 1 : 30; rabbit polyclonal antibodies to human γH2A.X (Abcam) at a dilution of 1 : 150; human antibodies to human centromere proteins (Sigma-Aldrich) at a dilution of 1 : 70 in PBT. The preparations were washed three times for 15 minutes in PBS with 0.1 % Tween-20 and incubated for 60 minutes at 37 °C with the following secondary antibodies, which were used in various combinations: donkey antibodies to rabbit immunoglobulins conjugated with a Cy3 fluorescent label (Jackson Laboratories, USA) at a dilution of 1 : 500; goat antibodies to mouse immunoglobulins conjugated with a fluorescent FITC label (Jackson Laboratories) at a dilution of 1 : 30; goat antibodies to rabbit immunoglobulins conjugated with a fluorescent FITC label (Jackson Laboratories) at a dilution of 1 : 150 and goat antibodies against human immunoglobulins conjugated with a fluorescent label AMCA (Vector Laboratories) at a 1 : 50 dilution. The preparations were washed in PBS, dried, and 15 µl of antifade solution was applied (Vectashield; Vector Laboratories) to prevent fluorescence quenching and covered with a cover glass.

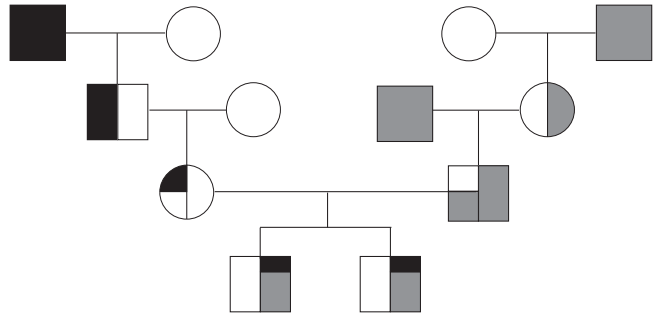

Fig. 1. Pedigree of the heterozygotes for translocation rob(OAM5;11).

The squares show males, the circles indicate females, the black fill shows argali genome, the gray shows mouflon genome, the white shows the Romanov sheep genome.

Microscopic analysis was performed at the Microscopy Center of the Siberian Branch of the Russian Academy of Sciences. The preparations were analyzed under Axioplan 2 microscope (ZEISS, Germany) equipped with a CCD video camera (CV M300, JAI Corporation, Japan), a set of CHROMA filter kits and ISIS4 image processing software (MetaSystems GmbH, Germany). Image brightness and contrast were edited using Corel PaintShop Photo Pro X3.

We analyzed 93 spermatocytes of the Romanov rams and 101 spermatocytes of the translocation heterozygotes. For analysis, we selected the cells at the pachytene stage, in which all autosomal bivalents of the standard *O. aries* karyotype were completely synapsed. Chromosome lengths and the relative position of MLH1 foci were measured using MicroMeasure 3.3 (Reeves, 2001).

To analyze the distribution of MLH1 foci along the chromosomes, we calculated the absolute position of each MLH1 focus, multiplying the relative position of each focus by the absolute length of the corresponding chromosome arm, averaged for all metacentrics. To construct a recombination map, we divided the chromosomal arms into equal intervals corresponding to 1 µm. For each interval, we calculated the proportion of MLH1 foci located on it out of the total number of foci located on a given chromosome.

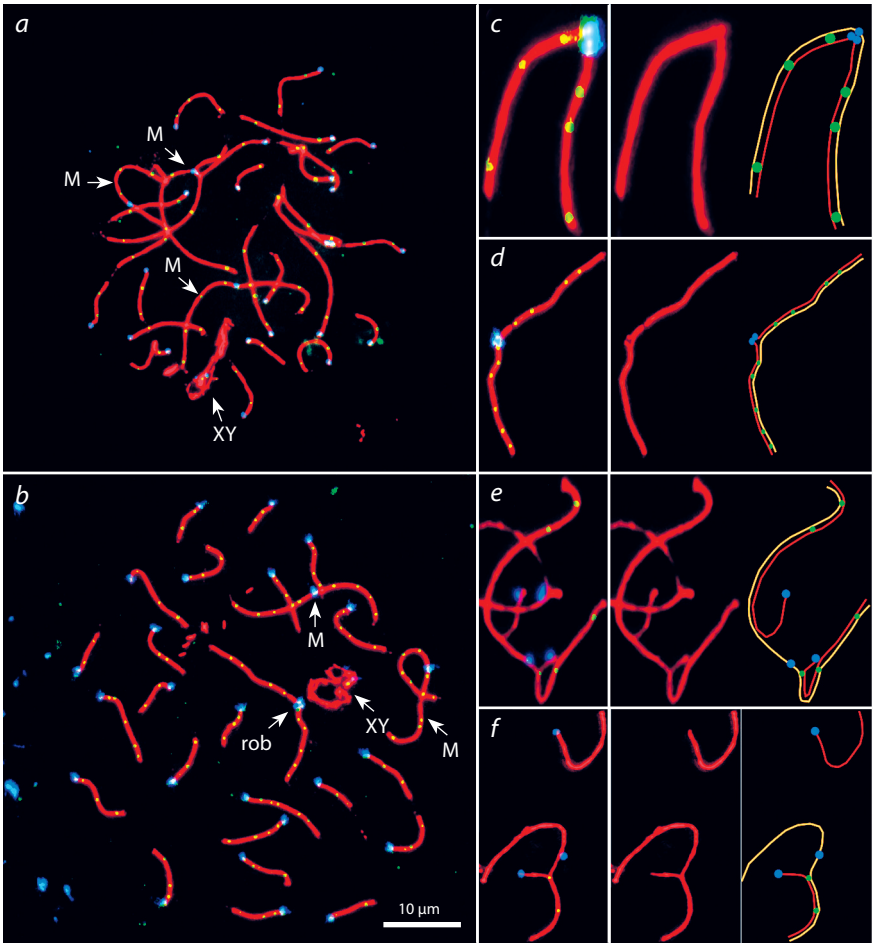

Fig. 2. Spermatocytes of Romanov rams (*a*), heterozygotes for rob(OAM5;11) (*b*) and magnified images of various synaptic configurations of the Robertsonian trivalent (*c–f*).

c – trivalent is completely synapsed; *d* – pericentromeric regions of acrocentrics are synapsed and form a side arm; *e* – the pericentromere region of trivalent is partially asynapsed; *f* – one of the acrocentrics in trivalent is completely asynapsed. First column shows merge of three color channels, second column shows the red channel only, third column shows a schematic image of the trivalent. The red signal represents SYCP3; green – MLH1; blue – centromere. M indicate metacentric bivalents, rob – Robertsonian trivalent, XY – sex bivalent.

We used ANOVA to test the effect of heterozygosity for the chromosomal rearrangement on the recombination characteristics of chromosomes involved. Statistical tests were performed using Statistica 6.0 (StatSoft). The average values of the SC lengths and the number of MLH1 foci are given with standard deviations (±SD).

Results

The karyotype of pachytene cells of Romanov rams contained three large metacentric bivalents, indistinguishable from each other, 23 acrocentric bivalents, forming a continuous series in descending length, and the sex bivalent (Fig. 2, *a*). Pachytene spermatocytes of the heterozygotes for the Robertsonian translocation contained two metacentric bivalents and trivalent formed by the sheep chromosome 3 and acrocentric argali homologs (Fig. 2, *b*). The trivalents did not differ in the average SC length from the bivalents of the two other metacentric chromosomes in heterozygotes ($t = 0.87$, $p = 0.38$), but they were shorter than the bivalents of all three metacentric chromosomes in the normal karyotype ($t = 5.36$, $p < 0.001$) (see the Table). Therefore, in the further analysis, we compared the recombination characteristics of the translocation trivalent with the combined data on the bivalents of two metacentric chromosomes in heterozygotes.

Figure 2, *c–f* present various variants of synaptic configurations found in the heterozygotes. The most frequent was the variant with almost complete pairing between metacentric chromosome 3 and its acrocentric homologues (see Fig. 2, *c*). Sometimes centromeres of acrocentrics superimposed on each other, forming a small lateral arm (*d*). Asynapsis of the pericentromeric regions of one or both acrocentrics was found in 5.0 ± 2.2 % of cells (*e*). The average size of the unpaired region was 24.2 ± 14.3 % of the trivalent length. In one case, we observed complete asynapsis of a longer acrocentric (*f*).

Immunolocalization of the phosphorylated form of histone H2A.X (γH2A.X) allowed us to visualize areas containing non-repaired DNA double-strand breaks (Fig. 3). At the leptotene stage, such areas were numerous and were present on all chromosomes (see Fig. 3, *a*). In the early pachytene, we observed γH2A.X signals on unpaired sites of autosomes, including the asynapsed areas of the acrocentric elements of the translocation trivalent (see Fig. 3, *b*). In rare cases, the entire arm of the trivalent, including its synaptic part, was subjected to epigenetic modification (see Fig. 3, *c*). By the end of the pachytene, the γH2A.X signal remained on the sex bivalent only and was absent on the trivalent (see Fig. 3, *d*).

Visualization of recombination nodules using antibodies to the MLH1 protein (see Fig. 2) allowed us to estimate the number of crossovers on the chromosomes of interest. We did not find differences in the average number of MLH1 foci at the Robertsonian trivalent with that at the bivalents of the two other metacentric chromosomes in the heterozygotes for rob(OAM5;11) ($t = 1.33$, $p = 0.18$) whereas the bivalents of the three metacentric chromosomes, including chromosome 3, in the normal homozygotes had fewer MLH1 foci ($t = 3.59$, $p < 0.001$).

The distribution of MLH1 foci along chromosome 3 of the translocation trivalent was similar to the distribution observed on the bivalents of metacentric chromosomes 1–2 in heterozygotes for rob(OAM5;11) and on the bivalents of chromosomes 1–3 in the standard karyotype (Fig. 4). It was relatively uniform. Unlike the chromosomes of many other mammals (Ruiz-Herrera et al., 2017), the metacentric chromosomes of the rams did not have pronounced peaks in the distal chromosome regions. The frequency of MLH1 foci in the

Recombination characteristics of the chromosomes 1–3 in the rams of the standard karyotype (+/+) and chromosomes 1–2 and 3 in heterozygotes for rob(OAM5;11) (+/rob)

Chromosomes	Number of chromosomes studied	Length of synaptonemal complex, μm	Number of MLH1 foci	Relative distance between adjacent MLH1 foci (chromosome length fraction)	Percent of MLH1 foci in pericentromeric regions*
1–3 (+/+)	279	35.8 ± 8.4	5.7 ± 1.2	0.18 ± 0.01	6.9 ± 0.6
1–2 (+/rob)	202	31.6 ± 8.3	6.0 ± 1.4	0.17 ± 0.01	5.8 ± 0.7
3 (+/rob)	101	30.7 ± 7.8	6.2 ± 1.4	0.16 ± 0.01	4.8 ± 0.9

* ± 2 μm from the centromere.

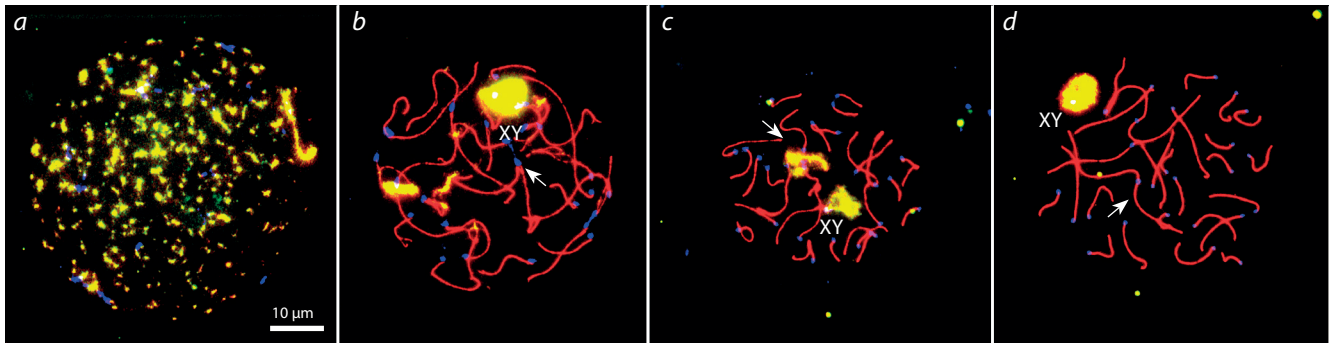

Fig. 3. Spermatocytes of rams heterozygous for rob(OAM5;11) at different stages of prophase I.

a, leptotene: γH2A.X signals are localized along the fragments of lateral SC elements; *b*, early pachytene: γH2A.X signals are localized at the asynapsed regions of autosomes and sex bivalent, whereas the trivalent does not show the signal; *c*, mid-pachytene: γH2A.X signals are localized at the asynapsed regions of the trivalent and sex bivalent; *d*, late pachytene: γH2A.X signal is localized at the sex bivalent only. Arrowhead indicate trivalent with translocation, XY – sex bivalent. Red signal represents SYCP3; yellow – γH2A.X; blue – centromere.

pericentromeric regions of trivalent and normal bivalents was reduced only in a short (1–2 μm) interval. However, we did not observe significant differences in the MLH1 foci number in pericentromeric regions ($p > 0.05$) between trivalent and two metacentric bivalents (see the Table). We also found no differences between trivalent and bivalents in the degree of crossover interference: the average distance between adjacent MLH1 foci was almost the same (see the Table).

Discussion

We found that rob(OAM5;11) heterozygotes showed a delayed synapsis of the Robertsonian trivalent in a small percentage of pachytene spermatocytes. This is in agreement with the results of electron microscopic studies of the sheep spermatocytes heterozygous for rob(6;24), rob(9;10) and rob(7;25) (Dai et al., 1994a, b). However, in the cited papers, the percentage of abnormal spermatocytes was higher than in our experiment. The carriers of these translocations showed rather high percentage of associations between the asynapsed regions of trivalent and the sex bivalent. We did not observe such associations. These differences in trivalent synapses in heterozygotes for translocations that are polymorphic within a species, and for translocations that distinguish different species, might occur due to either the methodological differences and/or the genetic properties of the chromosomes involved in these translocations.

Delayed synapsis in the pericentromeric region of trivalent lead to delays in repair of DNA double-strand breaks, which in turn caused epigenetic modification of this region: H2A.X histone phosphorylation at serine 139. Typically, such a modification of an unpaired chromatin results in transcriptional inactivation of genes localized in modified regions (Burgoyne et al., 2009; Turner, 2015). If such events occurred in pachytene spermatocytes of the rams heterozygous for the translocation, they affected only a small percentage of the cells. Even so, the pericentromeric chromosome regions are usually enriched in repeated sequences and contain few genes. Therefore, it is unlikely that inactivation of such areas may lead to germ cell death. In addition, in the spermatocytes at the late pachytene stage, we did not observe either asynapsed regions or signals of epigenetic modification. Most likely, asynapsis was replaced by a nonhomologous synapsis, and the modification of chromatin turned out to be reversible. We cannot exclude a possibility that the cells with the delayed synapsis were eliminated earlier and did not reach the pachytene stage. However, even if such elimination occurred, it affected only a small percentage of the germ cells and should not have affected the fertility of the heterozygotes.

The number and distribution of crossovers on the bivalents of normal metacentric chromosomes and on the translocation trivalent were similar. A rather common effect of heterozygosity for Robertsonian translocations is the distalization of the distribution of crossovers along the arms of the involved chromosomes (Dumas, Britton-Davidian, 2002; Borodin et al., 2008; Dumas et al., 2015). We did not detect such distalization in the rob(OAM5;11) trivalents. The degree of centromeric interference in the trivalents did not exceed that of homozygotes for the normal metacentric chromosomes. In a number of cells, we observed MLH1 foci in the close vicinity of the centromere (see Fig. 2, *c*, *e*, Fig. 4). In terms

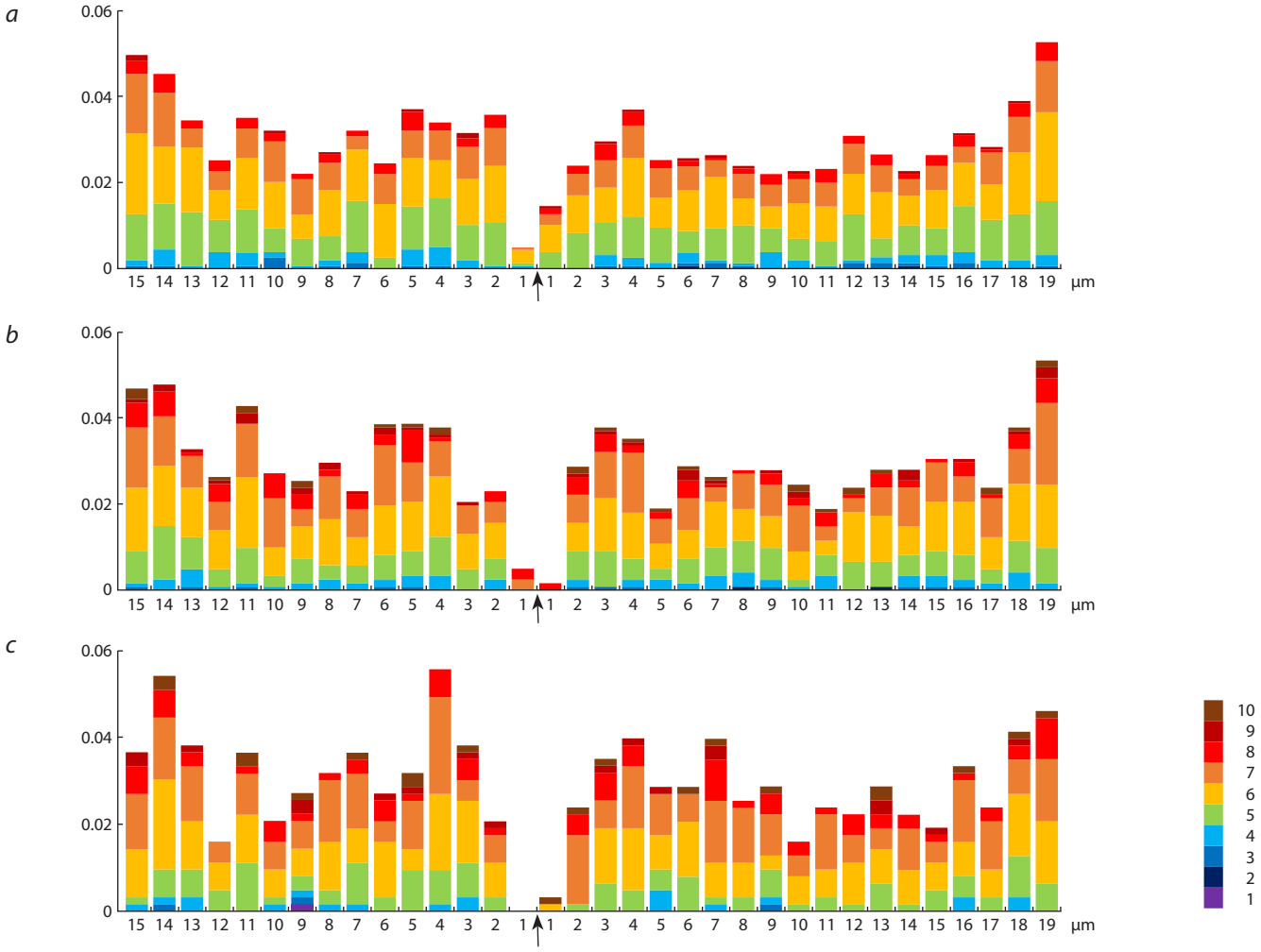

Fig. 4. Distribution of MLH1 foci along the bivalents of chromosomes 1–3 in normal homozygotes (*a*), along the bivalents of chromosomes 1–2 (*b*) and along trivalent chromosome 3 (*c*) in heterozygotes for translocation.

The X-axis shows the position of MLH1 foci at the bivalent relative to the centromere (indicated by arrow). The marks are equivalent to 1 μm. The Y-axis shows the proportion of MLH1 foci at each interval. The colors indicate the frequency of trivalents and bivalents containing different number of MLH1 foci within each interval, from 1 to 10.

of crossover interference, the translocation trivalent did not differ from normal bivalents either. Normal recombination in the Robertsonian trivalent ensures normal chromosome segregation and makes unlikely unbalanced gametes formation.

Conclusion

Thus, we found that heterozygosity for the evolutionary Robertsonian translocation involving chromosome 3 of domestic sheep and chromosomes 5 and 11 of argali does not cause significant changes in the key stages of meiosis and, therefore, should not lead to a decrease in fecundity in the offspring from interspecific hybridization of sheep.

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