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Male Horse Meiosis: Metaphase I Chromosome Configuration and Chiasmata Distribution

Ayman Al-Jaru[a, c]   William Goodwin[c]   Julian Skidmore[b]   Terje Raudsepp[d]

Kamal Khazanehdari[a]

[a]Molecular Biology and Genetics Laboratory, Central Veterinary Research Laboratory, and [b]Camel Reproduction Center, Dubai, United Arab Emirates; [c]School of Forensic and Investigative Science, University of Central Lancashire, Preston, UK; [d]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, Tex., USA

Abstract

Chromosome configurations and chiasma frequency during the metaphase I stage of spermatogenesis in the male horse are characterized in this work. The genome-wide frequency and distribution of chiasmata was detected as 49.45 ± 2.07 for 14 fertile stallions. All X and Y chromosomes shared a single chiasma at their pseudoautosomal region, while 1–4 chiasmata were observed in autosomal chromosomes. The chiasma frequency and distribution were further studied for 8 different bivalents identified by FISH in 5 fertile stallions. Genetic length was calculated from chiasmata data for the whole genome as well as for these 8 chromosomes. The findings complement the genetic linkage data and provide insight into the genetic basis of spermatogenesis in normal stallions.

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Studying the spermatogenesis of the horse, particularly meiotic division, is beneficial for the horse industry by identifying the causes of chromosomal abnormalities which result in embryonic loss, congenital abnormalities, and infertility. Meiosis is the most important division in spermatogenesis; it is a complex process during which homologous chromosomes pair and synapse, enabling the exchange of genetic material through meiotic recombination events. The physical location of meiotic recombination can be visualized as chiasmata at metaphase I (MI) which allow proper segregation of the maternal and paternal chromosome bivalent [Jones, 1984; Moens et al., 1987; Anderson et al., 1998; Barlow and Hultén, 1998; Anderson and Stack, 2005]. Moreover, chiasmata counteract the spindle microtubules pulling forces to ensure a proper alignment of the bivalents on the metaphase plate before their segregation to opposite poles [Turner, 2007]. Occurrence of chiasmata indicates that pairing, synapsis, and crossover have been successful during prophase I (PI). For proper segregation, at least 1 obligatory chiasma forms for each bivalent. Further, chiasmata are proportional to different factors, such as chromosome length and interference [Kaback et al., 1992; Anderson et al., 1998]. Failure of chiasma formation can cause non-disjunction of homologous chromosomes that may lead to chromosome abnormalities in the gametes [Hassold and Hunt, 2001].

Table 1. Description of 14 fertile horses examined

Horse ID	Date of castration	Age at castration, years	Type of anesthesia for castration procedure	Testis weight, g	
				right	left
H1	15/04/2007	3.5	local	190	195
H2	18/11/2007	3	general	200	210
H3	20/11/2007	6	local	185	195
H4	26/11/2007	3	general	205	190
H5	15/02/2008	5	local	210	220
H6	30/10/2008	3.5	local	200	190
H7	17/03/2009	4	local	215	230
H8	25/05/2009	3	local	210	244
H9	28/05/2009	3	local	362	310
H10	09/06/2009	6	local	283	291
H11	24/04/2009	4.5	general	290	295
H12	01/12/2009	4	general	122	140
H13	14/12/2009	4	general	175	180
H14	14/12/2009	3.5	general	200	190

The numbers and locations of chiasmata are studied at diakinesis/MI stages in which the chromosomes are condensed and become readily visible [Laurie and Hultén, 1985a; Barlow and Hultén, 1998; Sun et al., 2006]. These investigations provide a unique insight into chiasmata distribution and frequency as well as chromosomal configuration, contributing in estimating the genetic map interval.

The domestic male horse (2n = 64) has 31 autosomes, 13 are metacentric or submetacentric and 18 acrocentric. Among the sex chromosomes, the X is sub-metacentric and the Y is acrocentric or submetacentric [Bowling et al., 1997; Das et al., 2012]. Identification of individual chromosomes is more difficult at meiosis than mitosis because the chromosome configurations are more complex and centromeres are more difficult to visualize [Saadallah and Hultén, 1983]. Therefore, in order to facilitate MI karyotyping, FISH is the method of choice to identify metaphase chromosomes by using chromosome-specific fluorescently labeled probes. The combination of FISH and classical karyotyping allows the analysis of recombination frequencies and distributions for each specific pair of chromosomes [Hultén, 1974; Laurie and Hultén, 1985a, b].

Here, we investigated the meiotic chromosome configurations as well as chiasmata frequency and distribution at MI horse spermatocytes. Furthermore, for the first time, recombination maps and genetic length were estimated for 8 autosomes identified by FISH.

Material and Methods

Testicular samples were obtained from 14 fertile horses submitted to normal castration in the Dubai Equine Hospital, Dubai, United Arab Emirates or Sharjah Equine Hospital, Sharjah, United Arab Emirates. The castration procedures were carried out either under local or general anesthesia (table 1). All testis samples were examined for size (80–140 mm in length by 50–80 mm in width), weight (approximately 150–300 g), and appearance according to Amann [1993].

A small piece of tissue, ~1 cm³, was minced in Ham F10 media (Invitrogene, UK) within 30 min of collection. The tubular cells were squeezed out of the seminiferous tubules using 2 sharp curved forceps. The cell suspension was divided into 3 parts for different purposes, namely: (i) direct microscopic examination; (ii) the study of metaphase I, anaphase I, metaphase II (MII), and premeiotic metaphase by the air-drying technique; and (iii) storage in 10% glycerol at –80°C for future use.

Cells from the suspension were diluted 1:3 using normal saline (0.9% NaCl); one drop was placed on a clean slide and examined under a light microscope for the presence and motility of spermatozoa.

Genome-Wide Frequency and Distribution of Chiasmata
Meiotic chromosomes were prepared by the air-drying technique described by Hultén and Lindsten [1973] with some modifications. About 12 ml of freshly prepared, pre-warmed (37°C) 1% tri-sodium citrate (hypotonic solution) was added to the cell suspension drop-by-drop with gentle mixing after which the cells were incubated at 37°C for 20 min. At the end of hypotonic treatment, 1 ml of cold fixative (3:1 methanol:acetic acid) was added, and the cell suspension was centrifuged at 400 *g* for 10 min; the supernatant with the majority of the spermatozoa was discarded. The cells were fixed in fresh fixative and left on ice for 15 min. The fixative was changed 3–4 times at 15 min intervals, and cells were resuspended in an appropriate volume of fixative. One drop of the cell suspension was placed on a cleaned slide and air-dried at room temperature. The slides were stained in Giemsa stain (Merck, Germany) (1:20 Giemsa:phosphate buffer) at pH 6.8 for 2 min and examined under the light microscope for the presence of MI and MII cells as well as for metaphases of premeiotic mitotic spermatogonia.

The number of configurations and chiasma at MI were scored in 1,107 cells from 14 stallions, and the average number of chiasmata per bivalent and the total number of chiasmata per cell were calculated.

Identification of Meiotic Bivalents by FISH
Coordinates of the MI cells scored for chiasmata frequency and distribution from 5 different horses were recorded for subsequent FISH analyses. Therefore, the slides were destained in fixative and methanol for 5 min and air-dried. The slides were dehydrated in ethanol and overlaid with a probe cocktail containing biotin- or digoxigenin-labeled horse bacterial artificial chromosome (BAC) clones specific for chromosomes 2, 6, 10, 13, 15, 24, 26, and 31 (table 2) and hybridization buffer (Chrombios, Germany). Slides were placed on an 85°C hot plate for 6 min and incubated overnight at 37°C in a humidified chamber. The slides were washed twice in 2× SSC solution containing 50% formamide (Q-Biogene, USA) at 45°C, once in 2× SSC at 45°C, followed by further washes

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Table 2. Horse chromosome-specific probes for FISH

Chromo-some	Gene/marker	Cytogenetic location	Reference
2	*PNOC*	2q13	Raudsepp et al. [2008]
6	*NINJ2*	6q12q13	Raudsepp et al. [2008]
10	*PREP*	10q17	Perrocheau et al. [2006]
13	CH241-106P18 (BAC clone)	13q15q16	Raudsepp et al. [2008]
15	*LTBP1*	15q24	Raudsepp et al. [2008]
24	*CHGA*	24q16.2q16.3	Perrocheau et al. [2006]
26	*ROBO2*	26q14	Raudsepp et al. [2008]
31	*MAP3K4*	31q13	Perrocheau et al. [2006]

with 4× SSC containing 0.1% Tween 20 at 45°C and for a second time at room temperature for 5 min each. After blocking in 4× SSC containing 5% non-fat dry milk at room temperature for 5 min, the slides were incubated with streptavidin-FITC or anti-DIG TRITC at 37°C in a humidified chamber for 30 min. The slides were washed 3 times with 4× SSC solution containing 0.1% Tween 20 at room temperature for 10 min and mounted in antifade containing DAPI (Vectashield, Germany). The results were visualized under an Olympus BX61 fluorescence microscope (Olympus, Japan), and images were captured using the Applied Imaging Cytovision 3.1 software (Applied Imaging, UK). The localization and frequencies of chiasmata for the 8 labeled chromosomes were determined and analyzed using descriptive statistics, i.e. mean, range, SD, and inferential statistics (F test). Two-way and 3-way ANOVA was applied using SPSS (version 16) and Microsoft Excel (2007, Microsoft Corporation).

Results and Discussion

Different stages of spermatogenesis were observed in fresh preparations, namely: premeiotic mitotic metaphases (spermatogonial metaphases), PI, MI, and MII (fig. 1). Meiotic PI was the predominant cell type in meiotic preparations. This was expected as it is the most prolonged stage in meiosis [Parvinen et al., 1991]. A large number of spermatozoa were also observed at every preparation, indicative of the successful progression of previous stages of spermatogenesis.

Chiasmata Frequency and Distribution in All Chromosomes

A total of 1,107 Giemsa-stained MI cells from 14 stallions were photographed. The number of bivalents per cell was 32 with different chromosomal configurations. No uni- or multivalents were observed. All autosomal bivalents had at least 1 typically distally located chiasma.
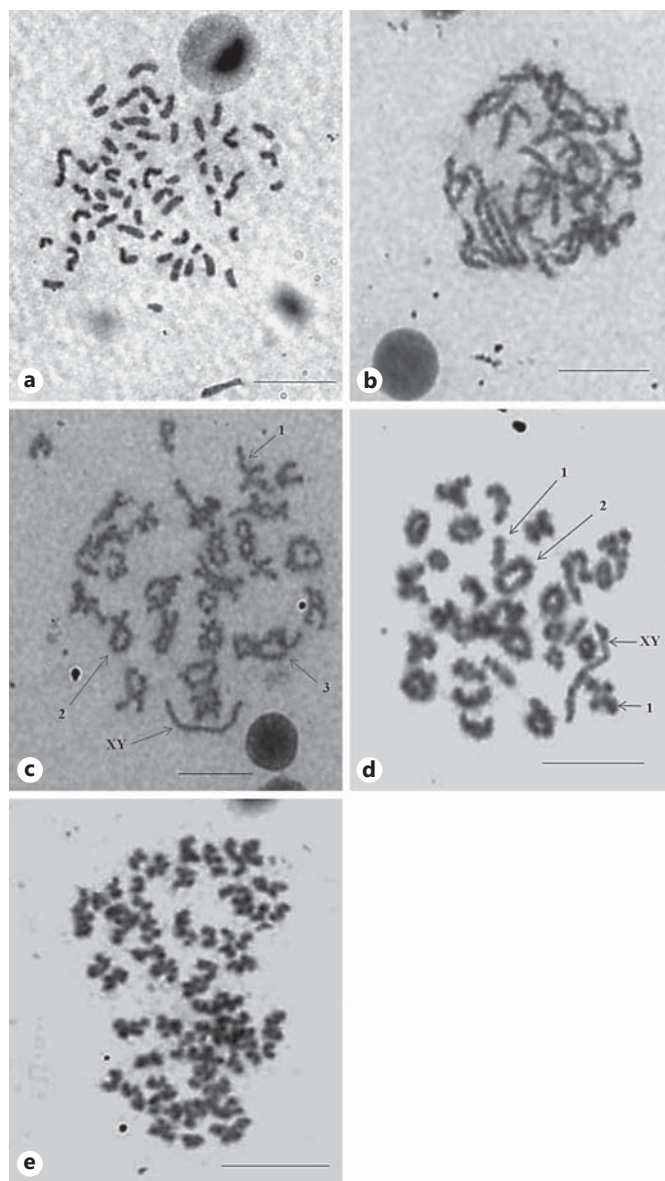

Fig. 1. Giemsa staining of different spermatogenetic cell stages from normal male horses. **a** Premeiotic mitotic metaphase, displaying 64 compacted chromosomes smaller than mitotic ones. **b** Prophase I. **c** Primary spermatocyte diakinesis. The homologous chromosomes show different configurations such as rod shape bivalent with 1 chiasma (1), bivalent with 2 chiasmata (2), bivalent with 3 chiasmata (3), and XY bivalent with 1 chiasma and rod shape (XY). **d** Primary spermatocyte MI with different configurations such as rod or cross shape bivalent with 1 chiasma (1), ring shape bivalent with 2 chiasmata (2), and XY bivalent with 1 chiasma and rod shape (XY). **e** MII with coiled, fuzzy, and twisted chromosomes. Scale bars = 10 μm.
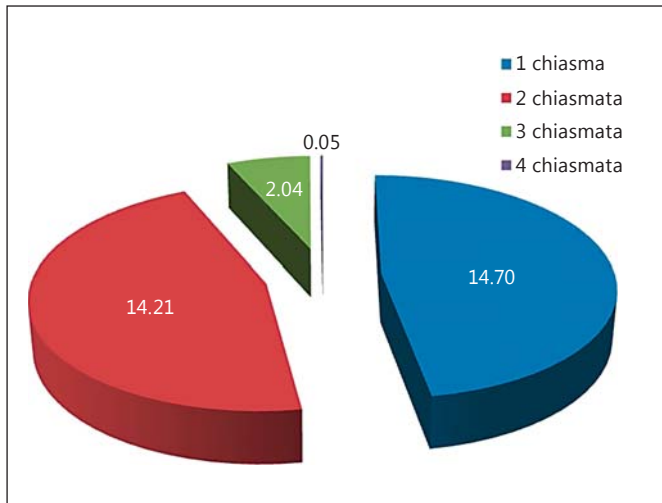
Fig. 2. Autosomal bivalent frequency with 1–4 chiasmata among 14 stallions (n = 1,107 cells).

Table 3. Mean chiasmata frequency of autosomal bivalents per nucleus among 14 stallions

Horse ID	Scored cells, n	Mean ± SD	Range
H1	60	50.08 ± 1.72	47–53
H2	72	50.67 ± 1.71	47–54
H3	73	48.78 ± 1.99	45–56
H4	77	49.19 ± 2.23	43–54
H5	63	49.70 ± 2.00	45–53
H6	63	49.92 ± 1.99	47–55
H7	114	48.45 ± 1.76	45–54
H8	80	49.11 ± 1.91	45–54
H9	106	48.76 ± 1.92	46–55
H10	94	50.18 ± 2.44	46–56
H11	83	49.25 ± 1.94	46–54
H12	79	49.68 ± 1.86	46–54
H13	69	49.80 ± 2.31	45–55
H14	74	49.64 ± 1.93	45–54
Total	1,107	49.45 ± 2.07	43–56

The XY bivalents had just 1 chiasma. Bivalents appeared as rod-, cross-, or ring-shaped, depending on the number and position of chiasmata (fig. 1).

The number of chiasmata was different between chromosomes and mostly correlated with chromosome length. Small chromosomes typically showed 1 chiasma, while longer chromosomes showed 2 or more chiasmata. The maximum number of chiasmata per chromosome was 4 and was only observed for chromosome 1.

The average number of autosomal chiasmata per nucleus (31 autosomes) ranged from 43 to 56 (49.45 ± 2.07, mean ± SD) with a mean of 1.63 chiasmata per autosomal bivalent (table 3). By including the XY bivalent, the total number of chiasmata per nucleus ranged from 44 to 57 (50.45 ± 2.07). Significant variation ($p < 0.05$) was observed in the mean chiasmata frequency for autosomal chromosomes among stallions, ranging from 48.45 ± 1.76 to 50.67 ± 1.71 with an inter-individual difference of 4.5%. The numbers of chiasmata per autosomal bivalent ranged from 1 to 4 and were as follows: 9–20 (14.7 ± 1.81; $p < 0.05$) bivalents with 1 chiasma, 7–22 (14.21 ± 1.93; $p < 0.05$) bivalents with 2 chiasmata, 0–5 (2.04 ± 0.84; $p < 0.05$) bivalents with 3 chiasmata, and 0–1 (mean = 0.05; $p = 0.045$) bivalents with 4 chiasmata (fig. 2). Significant heterogeneity ($p < 0.05$) among stallions was observed for the frequency of autosomes with 1, 2, 3, and 4 chiasmata.

The average number of chiasmata for all autosomal chromosomes in the male horse was 49.45; considering 1 chiasma per 50 centimorgan (cM), this corresponded to a genetic map length of 2,472.5 cM. Together with the 1 chiasma always present within the XY bivalent, the total length of the male horse genome is 2,522.5 cM, which is in good agreement with the 2,772 cM from linkage map data [Swinburne et al., 2006]. Given that in many organisms the length of genetic maps exceeds the genome length based on chiasma frequency [Sybenga, 1996], these findings in the horse are notable.

A comparison of the mean chiasma frequency in the horse and other species is shown in table 4. In male horses the mean frequency of chiasmata per nucleus reported in this study was 50.45 ± 2.07, which is less than reported by Scott and Long [1980] (54.40 ± 1.80 per cell). These differences could be attributed to several factors, such as different stallions, methodology, and research groups. Similarly, discordant results for chiasmata frequency in men are reported by 2 human studies (table 4).

The size of the horse genome is approximately 2.7 Gb which is close to the human (3.2 Gb) and other mammalian species [Wade et al., 2009]. The genetic map length presented here is very similar to that of humans which is 2,490 cM based on the chiasma count and 2729.7 cM by linkage data [Dib et al., 1996; Sun et al., 2004]. In addition, the total number of chiasmata in male horses is very close to that in human males, 50.61 ± 3.87 per cell, even though horses have more autosomes (31) than humans (22). This is likely because the horse has more acrocentric (18) and short chromosomes compared to humans (5 acrocentric

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Table 4. Comparison of mean average number of chiasmata (± SD) per nucleus for different species

Species	Average number of chiasmata	Scored cells	Reference
Horse, male	50.45±2.07	1,107	present report
Horse, male	54.40±1.80	221	Scott and Long [1980]
Human, male	50.61±3.87	41	Hultén [1974]
Human, male	45.30±4.52	91	Fang and Jagiello [1988]
Sheep, male	51.20±4.70	50	Longue [1977]
Goat, male	49.70±4.00	20	Longue [1977]
Bull	49.50±4.10	325	Longue [1977]

Table 5. Chiasmata frequency and genetic length in 8 individual chromosomes in 5 stallions

Chromo-some	Scored cells	Bivalent		Genetic length, cM	Linkage map, cM[a]
		range	mean ± SD		
2	73	2–3	2.67±0.47	133.5	128.8
6	87	2	2±0	100	126.8
10	96	2	2±0	100	105.8
13	63	1–2	1.35±0.48	67.5	58
15	73	2	2±0	100	96.7
24	72	1–2	1.08±0.28	54	47.2
26	80	1	1±0	50	24.4
31	74	1	1±0	50	41.1

[a] Swinburne et al. [2006].

Fig. 3. FISH analysis for chromosomes 6, 10, and 31 (green; FITC) and chromosomes 15 and 26 (red; TRITC). **a** Premeiotic mitotic metaphase (spermatogonial metaphase). **b**, **c** Primary spermatocyte metaphase. **d** Second meiotic metaphase. Scale bars = 10 μm.

chromosomes) which reduces the likelihood of having more chiasmata [Kaback et al., 1992].

The frequency of autosomal chiasmata in stallions is also similar to other domestic species such as sheep (51.20 ± 4.70) with 26 autosomes, goats (49.70 ± 4.00) with 29 autosomes, and bulls (49.50 ± 4.10) with 29 autosomes.

Chiasmata Frequency and Distribution per Selected Chromosomes

Eight different horse chromosomes, chromosome 2, 6, 10, 13, 15, 24, 26, and 31, were selected to study their chiasmata frequency and distribution (table 5). Those chromosomes were particularly selected since they represent different chromosomal groups with different size and centromere position: chromosomes 2, 6, 10, and 13 are submetacentric, 15, 24, 26, and 31 are acrocentric [Bowling et al., 1997]. After the numbers of chiasmata per chromo-
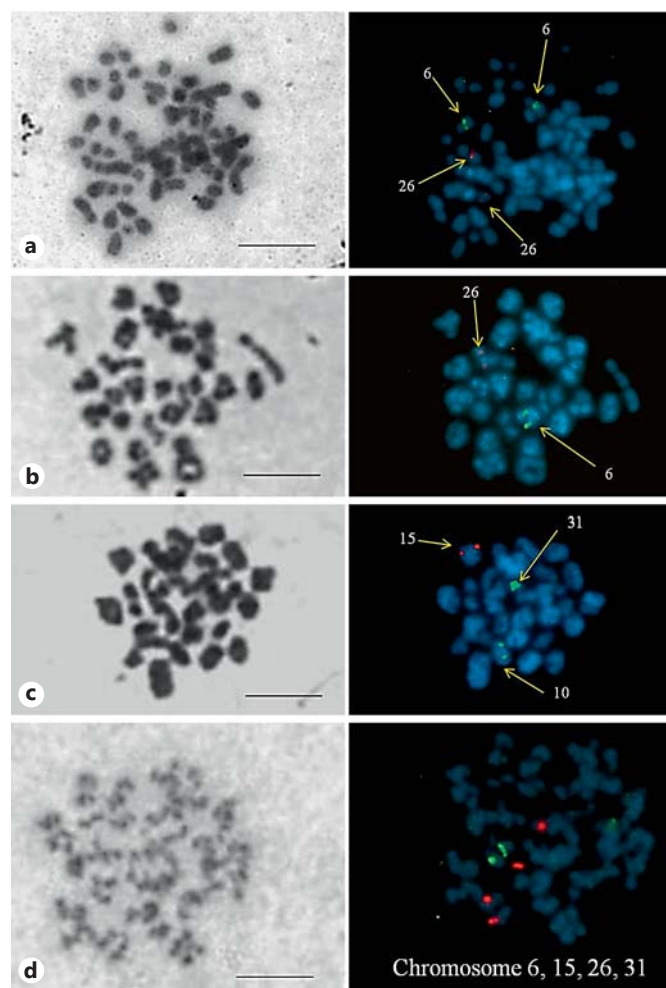
some and per cell were determined, FISH was performed to identify these chromosomes in the same cells (fig. 3). The quality of FISH signals and correct identification of chromosomes depended on the quality of cell preparations as well as the condensation of chromatin after fixation.

The number of chiasmata for chromosomes 2, 13, and 24 showed variation between cells but was not significantly different across the 5 stallions (p > 0.05). Chromosome 2 is large and submetacentric and showed 3 chiasmata, 1 in the p arm and usually 2 in the q arm in around 67% of the scored cells (73 cells). Chromosome 13 is the smallest submetacentric autosome and showed 1 chiasma in the q arm in around 65% of the scored cells (63 cells), whereas the single chiasma in the p arm was observed in only 35%

of the cells which could be due to the position of the first crossover that usually determines the number of extra foci on the same chromosome. The small acrocentric chromosome 24 had only 1 chiasma (very rarely 2) in 91.7% of the cells. Chromosomes 6, 10, and 15 had 2 chiasmata in all observations, while chromosomes 26 and 31 had only 1 chiasma near the telomere in all scored cells. In submetacentric chromosomes 6 and 10, 1 chiasma was identified in each arm, giving a ring-shaped chromosomal configuration. The 2 chiasmata in chromosome 6 were mostly located interstitially, while in chromosome 10 they had distal locations. On the other hand, the 2 chiasmata in the acrocentric chromosome 15 were commonly present in interstitial and distal locations, producing a ring shape with open-end chromosomal configuration. The genetic length for the 8 chromosomes was close to that obtained from the linkage map (table 5).

In summary, spermatogenesis, particularly meiotic, abnormalities can cause significant economic loss for horse breeders due to production losses as well as the cost of care for the stallion, mare, and foal. Meiotic errors can lead to sub- and infertility, most commonly due to nondisjunction of chromosomes in MI or MII resulting in aneuploidy, for instance sex chromosome aneuploidy (Klinefelter syndrome, 65,XXY), mosaic chromosomal configurations 64,XY/65,XXY or 63,X/65,XYY [Mäkinen et al., 2000; Lear and Bailey, 2008; Durkin et al., 2011], and autosomal trisomies, such as trisomy 28 (colt with small stature and azoospermia) or trisomy 23 (colt with multiple developmental defects) [Lear and Bailey, 2008], most of which may be explained by meiotic non-disjunction. Besides numerical abnormalities, autosomal translocations, such as 64,XY,t(1;30) [Long, 1996] or 64,XY,t(5;16+marker) [Durkin et al., 2011], reduce stallion fertility but maintain a normal phenotype. Additionally, many defects in meiosis can cause apoptotic spermatocytes or megalospermatocytes [Holstein et al., 2003].

In conclusion, knowledge of testicular function and understanding of the normal stallion spermatogenesis, particularly meiotic division, as well as developing an accurate method for assessing spermatogenesis are important for the industry and can also enhance our understanding of the genetic mechanisms underlying reproductive disorders in stallions. Therefore, this study provides information about recombination frequency in fertile stallions and complements the existing genetic linkage data. Moreover, for the first time, recombination maps and genetic length were estimated for 8 autosomes identified by FISH. These results, by using fertile stallions and documentation of the normal range of recombination, could be the cornerstone in understanding the genetic basis of normal spermatogenesis and the meiotic disturbances observed in infertile stallions due to structural chromosome abnormalities.

Further studies need to be carried out in order to establish a recombination map for all horse chromosomes as well as to study the recombination frequency in infertile cases. Both unusual numbers and distributions are expected to be observed in infertile male horses and translocation carriers. Also, more studies are required to elucidate the frequency of recombination as well as their physical distribution in female horses.

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