

Preparation and analysis of spermatocyte meiotic pachytene bivalents of pigs for gene mapping

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ABSTRACT

Well-spread meiotic pachytene bivalents were obtained by using the prolonged hypotonic treatment combined with high chloroform Carnory's fixative solution from cells of the testes of domestic pigs. Comparison in the division index and length of pachytene bivalents with metaphase chromosomes showed that those of the former are 5 times higher and 3.42(1.87-5.98) times longer than those of the latter. Comparative studies on chromomere maps of bivalents and mitotic chromosomal G-bands were conducted by using the chromosome 12 as an example. Sex vesicle and various shapes of synaptic sex chromosomes have been observed. Two-color PRImed IN Situ (PRINS) labeling has been conducted successfully on pachytene bivalents of pigs.

Key words: *pachytene bivalents, sex chromosome synapsis, two-color PRINS, microsatellites, pigs.*

INTRODUCTION

The cell culture technique is very important for the success of preparing mitotic chromosomes of pigs. Gene mapping on somatic chromosomes is an effective way for analyzing the genome of pigs. However, as the development of precise gene mapping on chromosomes, mitotic chromosomes prepared by cell culture for gene mapping have shown disadvantages, such as the longer time required for and complexity of preparation. Moreover, the length of the mitotic chromosomes is generally not long enough for high-resolution mapping. Although we may obtain high resolution chromosome preparation by the treatment with some reagents, such as methotrexate and 5-BrdU in the cell culture, it leads to the cell division index to be lower[1]. To prepare the pachytene bivalents from the spermatocytes is an alternative way to get the high-resolution chromosomes for gene

mapping. Preparation, gene mapping and PRImed IN Situ (PRINS) labeling on pachytene bivalents have already been conducted successfully in human, hamster and rice-field eels[2-5]. Based on these studies, here a new way for studying the chromosomes and physical gene mapping on meiotic bivalents of pigs is reported.

MATERIALS AND METHODS

Preparation of meiotic pachytene bivalents

The method was based on Yu et al[4] with a modified protocol. The sample came from fragments of testicular biopsy, followed by rinsing to remove blood substances in 0.85% NaCl, and stripping off the linked fat and external membrane. Then testicular and testis lobes were cut into multiple small segments(3-5mm) and were treated with hypotonic solution(0.45% sodium citrate and 0.075 mol/L KCl, 1:1 v/v) for 4-8 h at 37°C, fixed for 20 min in high chloroform Carnory fixative solution (chloroform, methanol and glacial acetic acid, 6:3:1-10:3:1, v/v), and twice refixed in another fixation (methanol and glacial acetic acid, 3:1, v/v) for 20 min. These segments then were cut into pieces for preparing the cells suspension. Then it was centrifuged at 800-1000 rpm for 10 min. The supernatant was discarded, and the pellet was carefully suspended in a small volume of supernatant residue. When the cell

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suspension was mixed evenly, 5-6 ml fixative solutions were added in it, and then they could be stored in the refrigerator. The next day, the spreads were made according to the air-drying technique on precooled slide. The air-dried slides can be stored (4°C) or stained immediately with 10% Giemsa solution.

Comparative studies on pachytene bivalents with mitosis metaphase chromosomes

Pachytene bivalents and mitosis metaphase chromosomes (prepared by blood cell culture) were observed under Olympus microscope. Division index (DI) was calculated. $DI = (\text{dispersion phase number} / \text{total cells number}) \times 100\%$. 10 photographs of well-spread pachytene bivalents and 10 of mitotic chromosomes enlarged with the same folds and the total length of autosomes were measured. Comparative studies on chromomere bands of bivalents and G-bands of mitotic chromosomes were conducted by using the chromosome 12 as an example.

Karyotype analysis and relative length of pachytene bivalents

The well-spread complete pachytene bivalents were selected for photographing and further analysis. According to the measurement of 10 cells, the relative length and standard errors of every autosomal bivalent were calculated. The relative length(%) = (length of individual bivalent/total length of 18 autosomal bivalents) $\times 100\%$. On the basis of every bivalent relative length, chromomere feature, and Reading Conference standardization [6], the karyotype of porcine pachytene bivalents was analyzed.

Preparation of plate of X, Y chromosomes synaptic process

Many spreads with various shapes of synaptic sex chromo-



Fig 1. well-spread pachytene bivalents of domestic pigs (1650 \times)

somes were photomicrographed. Sex chromosomes were arranged, clipped and pasted according to the period of synapsis.

Two-color PRINS

Two-color PRINS and detection were performed following Hindkjær et al[7], Koch et al[8-9] and Pellestor et al[10] with Dig-11-dUTP and Bio-16-dUTP used as the report molecules. Primers of GH gene were designed according to nucleotide sequences of the porcine GH gene (Vize et al)[11]. The oligonucleotide primers used were: forward 5' -CCAGCAGAGATCGGTTCAG-3' , reverse 5'-CATCCTCCAGCTCCTGCAA-3'. Specific oligonucleotide for porcine microsatellite SW60 were synthesized according to Rohrer et al[12]. The oligonucleotide primer sequences were: forward 5' -TCCGTATGCTGTGGATGTATC-3' , reverse 5' -CATGTTGCTGCAAATGGC-3' . GH and SW60 were labeled by Bio-16-dUTP and Dig-11-dUTP, respectively, in PRINS.

RESULTS

The results of preparation of pachytene bivalents of domestic pigs

A large amount of bivalent spreads can be obtained in the sex mature pigs (4-6 months). Well-spread meiotic pachytene bivalents can be obtained by using the prolonged hypotonic treatment along with the improved fixative treatment (Fig 1).

The results of comparative studies on pachytene bivalents with metaphase chromosomes

16344 meiotic cells have been observed and 4377 of them were in division phase. Division index of bivalents is 26.78%. 619 are in division phase among 11561 mitotic cells observed, and division index of mitotic chromosomes is 5.36%. Division index of bivalents is 5 times higher than that of mitotic chromosomes. The total length of autosomal bivalents is 285.77 ± 16.46 (n=10) in each division phase, while it is 83.47 ± 5.77 (n=10) in each mitosis phase. Length of bivalent is 3.42 (1.87-5.98) times longer than that of mitotic chromosome. Chromomere maps of bivalents are more abundant than mitotic metaphase G-bands and corresponded with mitotic early-metaphase G-bands, which were obtained by taking the chromosome 12 as an example (Fig 2).

The results of karyotype analysis of autosomal bivalents

Measurements indicate the haploidy karyotype of the pig is n=19 (18 autosome and 1 sex bivalent). The relative length of individual bivalent is basically

identical to that of mitosis chromosome measured by Liu[13]. The correlation coefficient (r) is 0.98 ($p < 0.001$). Our results show that the bivalents also can be divided into four groups, which are the same as the classification of porcine mitotic chromosomes by Reading Conference[6](Fig 3).

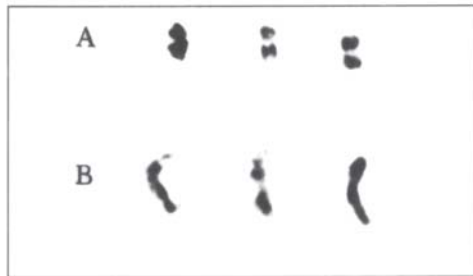


Fig 2. G-band of mitosis metaphase chr.12 and Chromomere maps of bivalent 12 (1650×)
A . Mitotic metaphase chr.12 **B.** Chromomere maps of meiotic bivalent 12

Tab 1. Comparison of relative length of bivalents and mitotic chromosomes of the domestic pig

Chr. No.	Bivalents (mean ± standard errors)	Mitotic chromosomes (mean)
1	10.78 ± 0.37	10.86
2	7.04 ± 0.12	6.14
3	6.54 ± 0.08	5.72
4	5.21 ± 0.07	5.24
5	4.67 ± 0.11	4.66
6	7.87 ± 0.11	6.53
7	4.94 ± 0.08	5.12
8	5.47 ± 0.08	5.39
9	5.65 ± 0.10	5.40
10	4.32 ± 0.11	3.81
11	3.51 ± 0.12	3.42
12	3.20 ± 0.11	3.14
13	8.57 ± 0.17	8.08
14	6.86 ± 0.09	6.11
15	5.95 ± 0.15	5.70
16	3.84 ± 0.13	3.70
17	2.87 ± 0.10	2.73
18	2.35 ± 0.08	2.54

Sex chromosomal synapsis

The results showed that sex chromosomes can be seen as sex vesicles before synapsis. They extend out of sex vesicle while they form synapsis and meanwhile X and Y chromosomes pair together and are also in the process of adjustment, then homologous

regions pair together, while non-homologous regions curve and fold autonomously. The morphology of the vesicles is irregular, hyperchromatic and ball-like after staining by Giemsa(Fig 4). This is quite similar to the results found in human bivalent and mouse synaptonemal complex studies [2],[14],[15]. Sex chromosomes can not be observed at the vesicle stage. The vesicles can be observed at the pachytene stage and show that the synapsis of X and Y chromosomes has a delay compared to the autosomes.

The result of two-color PRINS on bivalents

Two-color PRINS has been conducted successfully in the localization of SW60 (GH, red; SW60, yellow green)(Fig 5), and this confirms the regional localization result of SW60 by PRINS[16] and the localization of GH gene on somatic chromosome 12p12-p15[17].

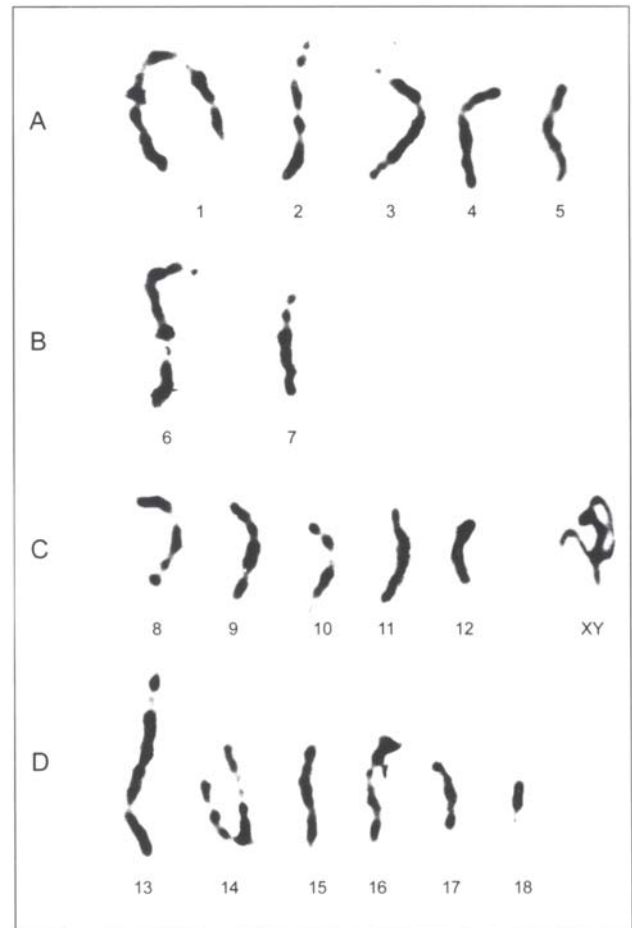


Fig 3. Karyotype of porcine spermatocyte pachytene bivalents (1650×)

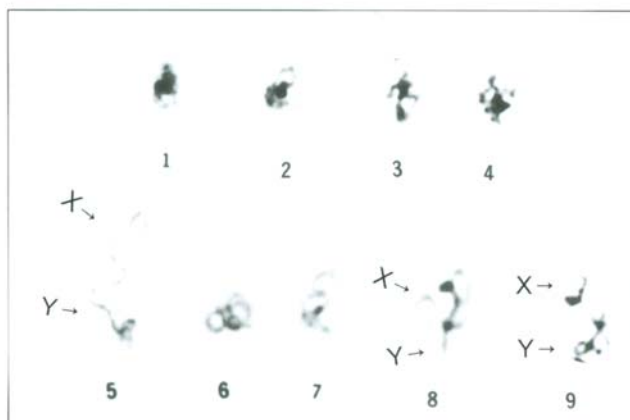


Fig 4. Synaptic process of porcine sex chromosomes(1650×)

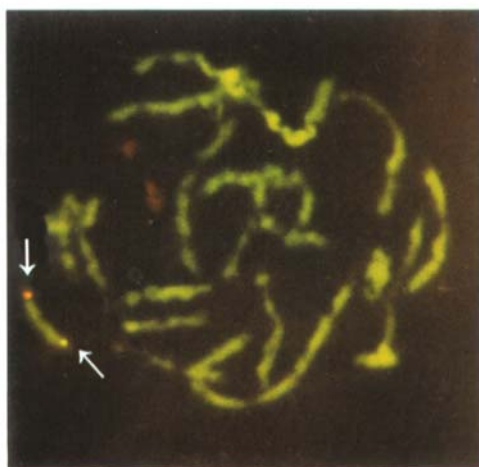


Fig 5. The spread after two-colour PRINS of GH and SW60 (825×)

Red colour: GH gene yellow green colour: SW60

DISCUSSION

Well-spread pachytene bivalents of the pigs can be obtained following the protocol presented in this paper. The complete porcine karyotype of bivalents has been analyzed for the first time. Our results showed that there are advantages of pachytene bivalents compared with the standard mitotic chromosomes. Firstly, it is an easy and simple method for obtaining large numbers of spreads compared with routine cell-culture techniques. Division index of bivalents is 5 times higher than that of mitotic chromosomes. Secondly, more precise physical mapping is possible due to the greater length of pachytene bivalents relative to mitotic chromosomes. Length of bivalents is 3.42(1.87-5.98) times than

that of mitotic chromosomes in this study. Thirdly, there is an improvement in the efficiency of detecting PRINS labeling and in situ hybridization due to the pairing of homologues and thus doubling of the DNA target. Finally, pachytene bivalents are more convenient for regional localization of genes or DNA markers without the process of multiply banding, because nature chromomere maps on the pachytene bivalents basically correspond with mitotic early-metaphase G-bands. Therefore, the preparation and PRINS of porcine pachytene bivalents will lay a foundation for high-resolution physical gene mapping in the pig.

Two-color PRINS of GH and SW60 on pachytene bivalents conformed the regional localization of SW60 because we already knew the precise localization of GH on the chromosome 12. The identification of a single copy gene by using very short sequence obtained from PRINS technique, especially two-color PRINS opened the possibility of a new strategy in the physical mapping of chromosomes. The method can offer the possibility of localizing genes by designing PRINS primers according to their sequences. Moreover, two genes can be detected with two different colors simultaneously by two-color-PRINS and the physical frame mapping of chromosome can be carried out directly.

The study of synaptic process of homologous chromosomes including sex chromosomes in prophase I of meiosis is of great importance to Biology, Genetics and Medicine. However, the synaptic process analysis of porcine sex chromosomes only focused on the ultrastructure of synapsis-the synaptonemal complexes. The paper is the first report about porcine sex chromosomes pairing behaviors based on the analyses in bivalents by using optical microscope. This study may provide more useful information for further research of porcine sex chromosomes.

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