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ORIGINAL ARTICLE

Cytogenetic comparisons between A and G genomes in *Oryza* using genomic *in situ* hybridization

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The genomic structures of *Oryza sativa* (A genome) and *O. meyeriana* (G genome) were comparatively studied using bicolor genomic *in situ* hybridization (GISH). GISH was clearly able to discriminate between the chromosomes of *O. sativa* and *O. meyeriana* in the interspecific F_1 hybrids without blocking DNA, and co-hybridization was hardly detected. The average mitotic chromosome length of *O. meyeriana* was found to be 1.69 times that of *O. sativa*. A comparison of 4,6-diamidino-2-phenylindole staining showed that the chromosomes of *O. meyeriana* were more extensively labelled, suggesting that the G genome is amplified with more repetitive sequences than the A genome. In interphase nuclei, 9-12 chromocenters were normally detected and nearly all the chromocenters constituted the G genome-specific DNA. More and larger chromocenters formed by chromatin compaction corresponding to the G genome were detected in the hybrid compared with its parents. During pachytene of the F_1 hybrid, most chromosomes of A and G did not synapse each other except for 1-2 chromosomes paired at the end of their arms. At meiotic metaphase I, three types of chromosomal associations, i.e. *O. sativa*-*O. sativa* (A-A), *O. sativa*-*O. meyeriana* (A-G) and *O. meyeriana*-*O. meyeriana* (G-G), were observed in the F_1 hybrid. The A-G chromosome pairing configurations included bivalents and trivalents. The results provided a foundation toward studying genome organization and evolution of *O. meyeriana*.

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Introduction

The genus *Oryza* is composed of two cultivated and more than 20 wild species, and 10 genome sets have been identified including the diploids and tetraploids [1-3]. Wild rice species are extremely valuable gene resources for the genetic improvement of cultivated rice and for the study of genomics [4]. A better understanding of the genomic relationships among the species will facilitate effective conservation and efficient utilization of the gene resources in *Oryza* [5].

Genomic *in situ* hybridization (GISH) is a powerful method for the characterization of genomes and chromosomes in allopolyploid species [6-8], hybrid plants [9, 10] and recombinant breeding lines [11, 12]. It has played a significant role in understanding genome origin and evolution in the genus *Oryza* [5, 13-17]. GISH was used to study relationships among the B, C and D genomes, and the result suggested that the D genome was closer to the C than to the B genome [13]. The differentiation and relationships among the C, D and E genomes in the *O. officinalis* complex were detected by multicolor GISH, which indicated that the C,

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Abbreviations: GISH (genomic *in situ* hybridization); DAPI (4,6-diamidino-2-phenylindole); FISH (fluorescence *in situ* hybridization); PBS (phosphate-buffered saline); NOR (nucleolar organizing region)

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D and E genomes shared a substantial amount of similar sequences, and differentiation between the D and C genomes of *O. alta* was less than that between the E genome and each of the C and D genomes [5]. The different *Oryza* genomes, A, B and C, involved in rice somatic hybrids, and A and C in hybrid plants, were distinguished using GISH technique [14-16]. Multicolor GISH was also applied to identify the genomic constitution of three tetraploid species ($2n$=4x=48) in the *O. officinalis* complex, i.e. *O. malampuzhaensis*, *O. minuta* and *O. punctata* [17].

O. meyeriana is a diploid wild rice species with G genome [2] and distributes in the tropics and subtropics of South and Southeast Asian countries [18]. It is one of three native wild rice species in China growing in the southern Yunnan Province and the southwestern Hainan Province [19, 20]. A variety of beneficial traits, such as disease and pest resistances, and tolerances to drought and shade stress, have been found in *O. meyeriana* [18, 21]. Efforts have been devoted to understanding the genomic composition [2] and genetic variation [3, 19, 20] of *O. meyeriana.* However, the cytological features of *O. meyeriana* and the genomic relationship between A and G genomes have not been thoroughly investigated.

In this study, we applied comparative GISH to distinguish between *O. sativa* and *O. meyeriana* chromosomes in their interspecific hybrid, and analyzed the spatial distribution and organization of the two genomes in interphase nuclei. In addition, we also investigated the intergenomic pairing at pachytene and meiotic metaphase I of the hybrid. These data provided a foundation toward studying genome organization and evolution of *O. meyeriana*.

Materials and methods

Plant materials and probes

O. meyeriana, *O. sativa* subsp. *indica* cv IR36 and their interspecific hybrid [22] were the target materials used for chromosome preparation. The 45S rDNA subclone pTa71, which contains the coding sequences for the 45S rRNA genes of wheat [23], and the total genomic DNAs of *O. sativa* and *O. meyeriana* were used as probes in fluorescence *in situ* hybridization (FISH) analysis.

Chromosome preparation

Plants were grown in a greenhouse. Root tips from vigorously growing plants were collected and fixed immediately in ethanol:acetic acid (3:1) at 4 °C overnight. After washing with distilled water, the root tips were treated with a mixture of 2% pectinase (SERVA) and 2% cellulase (SERVA) at 28 °C for 3 h before squashing on slides and drying in a flame [24]. For pachytene chromosome preparation, young panicles of plants were harvested and fixed. Microsporocytes at the pachytene stage were macerated in 2% pectinase and 2% cellulase at 28 °C for 2 h and squashed on slides. The slides were kept under -20 °C before FISH.

FISH

Plasmid DNA was isolated following an alkaline lysis method [25]. Total genomic DNAs of *O. sativa* and *O. meyeriana* were extracted from leaves according to the procedure of Doyle [26]. FISH was performed as described previously by Xiong *et al.* [27]. The probes were labelled with digoxigenin-11-dUTP or biotin-11-dUTP, following the protocol for nick translation suggested by the kit maker (Sino-American Biotechnology Company). The biotin-labelled probes were detected in a three-step detection/amplification: streptavidin-Cy3 (Vector Laboratories), biotinylated anti-streptavidin (Vector Laboratories) and streptavidin-Cy3. The digoxigenin-labelled probes were detected with sheep-anti-digoxigenin-FITC (Roche Molecular Biochemicals) and amplified with rabbit-anti-sheep-FITC (Vector Laboratories). For each step of the reaction, slides were incubated at 37 °C for 30 min and washed with phosphate-buffered saline at intervals. Finally the slides were counterstained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) in Vectashield, an antifade solution (Vector Laboratories).

Cytological measurements and analysis

Chromosomes were viewed with a fluorescence microscope system (Olympus BX60 equipped with a cooled CCD camera Sensys 1401E). Gray scale images were captured for each fluorescence channel and then merged with V^{++} Precision Digital Imaging System software. Digital images were measured using Meta Imaging Series 4.6 software and final images were integrated with Adobe Photoshop v5.1. For comparison of the lengths of the chromosomes between *O. meyeriana* and *O. sativa,* chromosomes in five complete mitotic cells of the F_1 hybrid were measured and standard deviations were calculated.

Results

GISH analysis of the F_1 hybrid of O. sativa × O. meyeriana

The biotin-labelled *O. meyeriana* genomic DNA as probes without blocking DNA of *O. sativa* was hybridized to mitotic metaphase chromosomes of the F_1 hybrid between *O. sativa* and *O. meyeriana*. All the chromosomes of the G genome were labelled with strong red signals and signals were hardly detected on those of the A genome(Figure 1A).

Bicolor FISH was performed on mitotic chromosomes of the *O. sativa* × *O. meyeriana* F_1 hybrid (Figure 1B) using

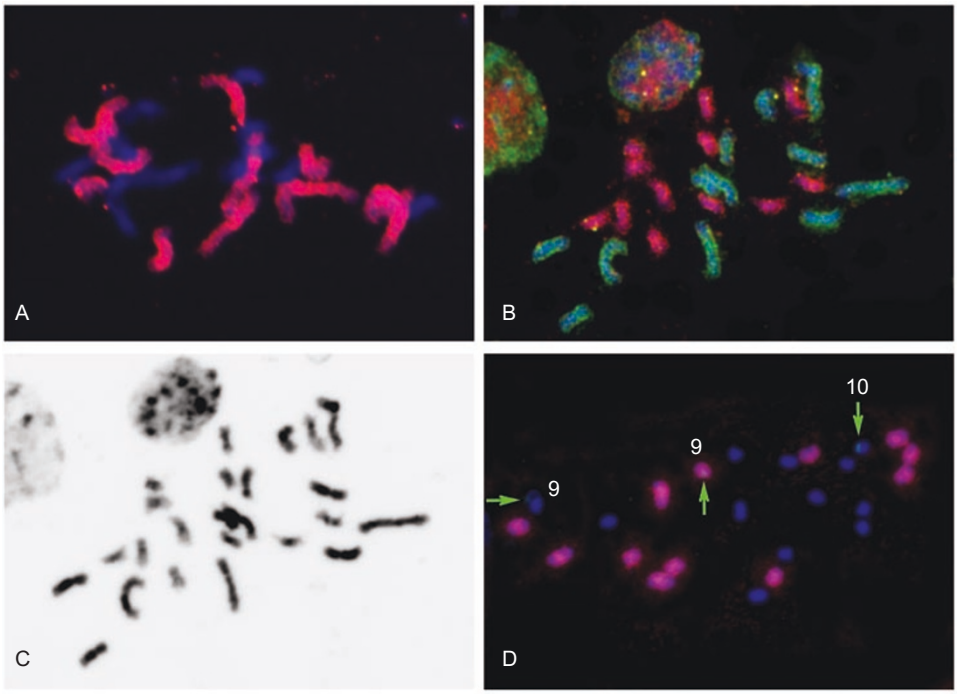

Figure 1 Bicolor GISH with the two genomic probes of A (red) and G (green) at mitotic metaphase of interspecific hybrid. Counterstaining was applied with DAPI (blue). **(A)** Mitotic chromosomes probed with the genomic DNA of *O. meyeriana* (red). **(B)** Discrimination of the two different genomes in mitotic metaphase. **(C)** B with DAPI-stained chromosomes was converted to a black-and-white image to enhance the visualization of the distribution of euchromatin and heterochromatin in chromosomes of metaphase. **(D)** Mitotic chromosomes probed with genomic DNA from *O. meyeriana* (red) and 45S rDNA (green). Arrows indicate the position of NORs.

the digoxigenin-labelled genomic DNA probes from *O. meyeriana* and biotin-labelled ones from *O. sativa*. Two kinds of hybridization signals were mutually exclusive and almost no mixed signals were observed. Twelve chromosomes appeared green and should be from *O. meyeriana;* the other 12 appeared red and should be from *O. sativa*. Thus, the A and G genomes were clearly discriminated on the same chromosome preparation.

Table 1 Comparison of length for corresponding chromosomes between *O. meyeriana* and *O. sativa*

Chromosome No.*	*O. meyeriana* (μm)	*O. sativa* (μm)	Ratio**
1	6.42±0.39	3.6 ±0.40	1.78
2	5.42±0.62	2.97±0.31	1.82
3	5.06±0.84	2.76±0.30	1.83
4	4.86±0.75	2.51±0.19	1.94
5	3.86±0.24	2.37±0.22	1.63
6	3.67±0.26	2.24±0.28	1.64
7	3.48±0.23	2.09±0.23	1.67
8	3.21±0.32	2.01±0.20	1.60
9	3.0 ±0.30	1.91±0.19	1.57
10	2.61±0.30	1.83±0.21	1.43
11	2.47±0.19	1.67±0.16	1.48
12	2.34±0.21	1.51±0.13	1.55
Total	46.4 ±3.44	27.47±2.70	1.69

*Chromosomes were numbered and arranged according to their length.
**O. meyeriana/O. sativa

DAPI binds preferentially to AT-rich regions [28] and is a common dye to stain heterochromatin [29]. Scrutiny of a number of cells at metaphase indicated that the chromosomes of *O. meyeriana* showed heavier DAPI stain than those of *O. sativa.* To enhance the visualization of distribution of euchromatin and heterochromatin along the mitotic chromosomes, Figure 1B with DAPI-stained chromosomes was converted to a black-and-white image (Figure 1C). For *O. sativa,* most of the notable heterochromatic regions were observed in the pericentric region and relatively low DAPI staining intensity was distributed in the distal chromosome arms. Unlike *O. sativa*, the entire chromosomes of *O. meyeriana* were heavily stained with DAPI.

GISH images clearly demonstrated that the hybrid cells showed an obvious difference in chromosome size between the A and G genomes. The chromosomes from *O. meyeriana* appeared much larger than those from *O. sativa* (Figures 1B and C). The chromosomes of the hybrid were

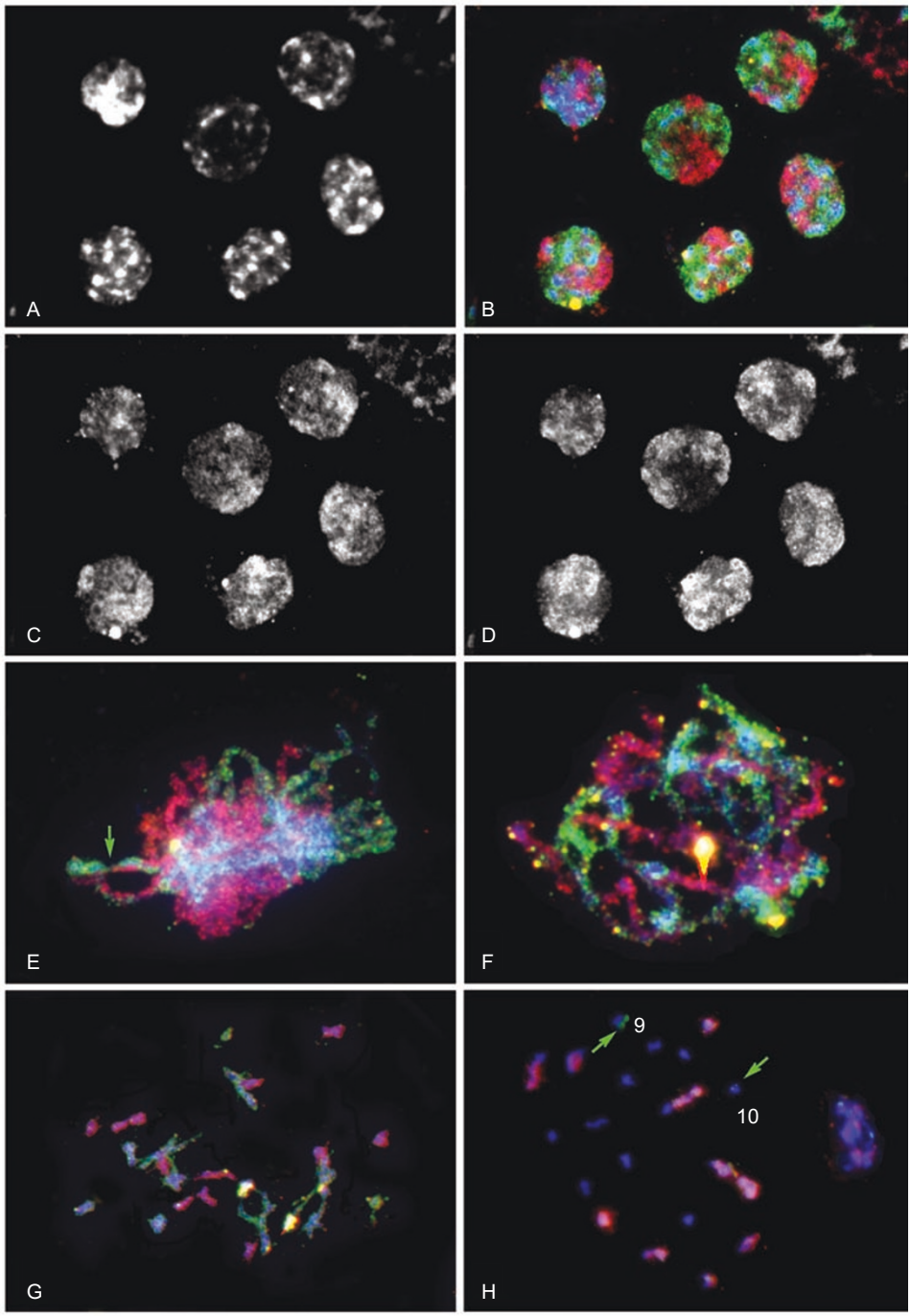

Figure 2 GISH on interphase nuclei and meiotic chromosomes of the interspecific hybrid. Chromosomes counterstained with DAPI. **(A)** Interphase nuclei stained with DAPI showing large and heavy stained chromocenters. **(B)** Discrimination of the two different genomes in interphase nuclei.The images of **A**, **C** and **D** were combined. **(C)** Distribution of the A genome within the nuclei. **(D)** Distribution of the G genome within the nuclei. **(E, F, G)** Multicolor GISH with the two genomic probes of A (red) and G (green) on pachytene, diplotene and meiotic metaphase I. **(H)** Meiotic metaphase I chromosomes probed with genomic DNA from *O. meyeriana* (red) and 45S rDNA (green). Arrows indicate the position of NORs.

measured and arranged in descending order of length (Table 1). The chromosomes from *O. meyeriana* and *O. sativa* were compared based on the assumption that karyotypes were similar between corresponding chromosomes among different species of *Oryza* [30, 31]. According to Table 1, we could reach the conclusion that the total chromosomal

length of *O. meyeriana* was 1.69 times that of *O. sativa*. Each chromosome of *O. meyeriana* was elongated compared with the corresponding one of *O. sativa*, but not the same in the elongated ratio for different chromosomes. Chromosomes 4 and 10 carrying nucleolar organizing regions (NORs) were the most and the least elongated chromosomes of *O. meyeriana*, respectively.

The 45S rDNA has been reported to locate on chromosomes 9 and 10 of *O. sativa* L. subsp. *indica* [32]. One locus of 45S rDNA was detected on chromosome 9 of *O. meyeriana* (unpublished data). FISH was also performed on mitotic metaphase chromosomes of the hybrid with probes of 45S rDNA and genomic DNA from *O. meyeriana*. The result showed that one 45S rDNA site was located on the G genome and two on the A genome (Figure 1D).

Genome spatial distribution and organization in interphase nuclei of the hybrid

In interphase nuclei of the hybrid, heterochromatin is organized as clearly distinguishable chromocenters, which are visible as bright, fluorescent domains after DAPI staining (Figure 2A). In general, 9-12 chromocenters were detected per nucleus. Bicolor GISH with the two parental genomic probes revealed that nearly all the chromocenters corresponded to the G genome-specific DNA. In addition, each of the two parental genomes tended to occupy a separate domain in the nucleus (Figure 2B-2D).

The distribution of the number of chromocenters per nucleus was compared between the hybrid and its parental species (Figure 3). In the nuclei of *O. sativa*, usually a few prominent chromocenters formed or the formed chromocenters were small. In *O. meyeriana*, the number of chromocenters varied widely in different nuclei and they were smaller and fainter than those in the hybrids (data not shown).

Chromosome pairing in the hybrid

The ability to differentiate the chromosome sets in the interspecific hybrid by GISH has provided new information on the interaction between homoeologous chromosomes during meiosis. Figure 2E showed a typical example from several hundred mid-pachytene nuclei of the diploid hybrid. At this stage, most chromosomes from *O. sativa* and *O. meyeriana* existed as univalent and were not associated with each other. Only one or two homoeologous pairings between the A genome and G genome per nucleus were detected at the terminal regions of chromosome arms. At diplotene, most chromosomes from different genomes were clearly discernible without intertwist. However, it is interesting that the hybrid showed low frequencies of bivalents among *O. sativa* chromosomes and among *O. meyeriana* chromosomes (Figure 2F). At meiotic metaphase I, three types of chromosomal associations were distinguished by GISH (Figure 2G), i.e. *O. sativa-O. sativa* (A-A), *O. sativa-O. meyeriana* (A-G) and *O. meyeriana-O. meyeriana* (G-G). The A-G chromosome pairing configurations observed in the hybrid included bivalents and trivalents (Table 2). Using 45S rDNA as a chromosome marker, there was no pairing to be detected between chromosomes carrying NOR (Figure 2H).

Table 2 Average values of different chromosome associations observed at meiotic metaphase I of the hybrid

No. of cells	Univalents		Bivalents		Trivalents	
	A	G	A-A	G-G	A-G	A-G
20	5.8	3.8	1.6	1.8	1.2	2

Discussion

The GISH method has been used to study differentiation and relationships among the A, B, C, D and E genomes of the genus *Oryza* [5, 13-17]. The different hybridization stringencies and the rates of blocking DNA used in GISH procedures, and the fluorescence intensities observed be-

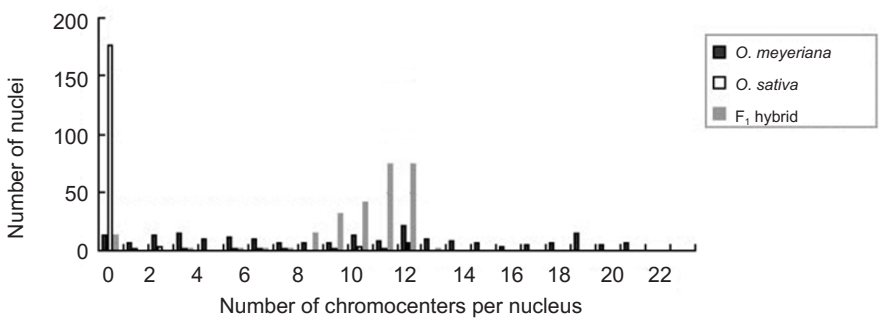

Figure 3 Distribution of the number of chromocenters per nucleus in the interspecific hybrid and parental species.

tween interspecific chromosomes within a nucleus provide phylogenetically important information for elucidating relationships among species in *Oryza*. GISH identifies A and C genomes in the F_1 hybrid between *O. sativa* and *O. officinalis* using 16 folds blocking DNA [16]. A comparison of the fluorescence signal intensity between C and D genomes within *O. latifolia*, and B and C genomes in *O. minuta*, indicated that co-hybridization existed between C and D genomes and B and C genomes [13]. Under the different stringent washing conditions, the multicolor GISH results indicated that the C, D and E genomes shared a substantial amount of similar sequences [5]. In the present study, GISH was clearly able to discriminate between the chromosomes of *O. sativa* and *O. meyeriana* in the F_1 hybrid without blocking DNA, and co-hybridization was hardly detected. This result clearly showed considerable divergence of the middle to highly repetitive DNA sequences and confirmed the wide genetic differences between the two species. These observations are in agreement with the results that *O. meyeriana* is considered as the most distinct species from those with an A genome and occupies the most basal position in the phylogeny of the genus [2, 3].

The quantity of repetitive sequence is the main factor that determines the genome size of similar ploidy [33, 34]. Using FISH technique, Uozu *et al.* [35] analyzed the distribution patterns of genome-specific repetitive DNA sequences in the genus *Oryza*, except for the G genome. They concluded that the variation in genome size and chromosome morphology is caused by repetitive sequences. According to our GISH results, the genomes of *O. sativa* and *O. meyeriana* were evidently different in chromosome length and heterochromatin contents. Obviously, the increase in genome size of *O. meyeriana* was mainly due to the amplification of repetitive sequences. This result is consistent with the conclusion reported by Aggarwal *et al.* [2], who demonstrated that the uniqueness of the G genome is due to the species-specific highly repetitive DNA sequences by using total genomic hybridization. Furthermore, because the whole chromosomal regions of *O. meyeriana* were evenly enhanced with DAPI stain (Figure 1C), our results proved that the chromosomes of *O. meyeriana* had been extended and evenly enhanced with repetitive sequences during the genome evolution.

The sizes of the A, B, C, E and F genomes of the diploid *Oryza* species were determined by flow cytometry and image analyses, and the results demonstrated that the total chromosomal length correlated well with the nuclear DNA content [35]. We also estimated the DNA content of *O. meyeriana* according to chromosomal length in the F_1 hybrid. This estimation was based on the assumption that (1) the haploid genome size of *O. sativa* is 430lMb and (2) the euchromatic and heterochromatic regions contain equal amounts of DNA per micrometer of chromosome length. However, the DNA content is usually underestimated for relatively heterochromatic chromosomes [36]. The DNA content of *O. meyeriana* might be more than 727lMb/1C because the species contains a large amount of heterochromatin.

Cytogenetic methods were used to detect the relationships among the genomes of the *Oryza* species, based on the observation of chromosome pairing behavior at meiotic metaphase I of interspecific hybrids [37-40]. However, chromosome pairing could not discriminate easily the genome constitution of the bivalents in hybrids. Multicolor GISH, using two different genomic probes simultaneously, not only gave high resolution in identifying the chromosome pairing between different genomes in an interspecific hybrid from pachytene to meiotic metaphase I but also provided a direct approach for characterizing genomic differentiations at both chromosomal and DNA levels. Our results showed that multicolor GISH could bring a better result than traditional cytogenetic methods.

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