

Full Length Research Paper

Assessment of genomic relationship between *Oryza sativa* and *Oryza australinesis*

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Accepted 6 January, 2010

The genomic relationship between *Oryza sativa* (2n = 24 AA) and *Oryza australinesis* (2n = 24 EE) has not been established. Genomic relationship between these two species was assessed by using three strategies: genomic *in situ* hybridization (GISH), meiotic chromosome pairing, pollen and spikelet sterility. The hybrid was produced between these two species at the International Rice Research Institute using embryo rescue technique. The chromosome pairing was examined in pollen mother cells of *O. australinesis*, *O. sativa* and the hybrid between *O. sativa* and *O. australinesis*. The hybrid was highly sterile with pollen stain ability being 0.05%. Both parents showed regular meiosis with normal chromosome pairing. The F1 hybrid exhibited limited chromosome pairing. On an average, 0 - 4 bivalents and 16 - 24 univalents were recorded at metaphase-1. The most frequent configuration was two bivalent and twenty univalent. The chromosomes of *O. australiensis* appeared larger and darkly stained. For genomic *in situ* hybridization, genomic DNA from *O. australiensis* was used as probe for the mitotic and meiotic chromosomes of the hybrid between *O. sativa* and *O. australiensis*. GISH revealed unequivocal discrimination of *O. australiensis* chromosomes that appeared yellow due to hybridization signal from *O. sativa* chromosomes that fluoresced red due to counterstaining with propidium iodide (PI). No cross hybridization was examined between the labeled genomic DNA of *O. australiensis* and the chromosomes of *O. sativa*. The paired chromosomes were discriminated as *autosyndetic* and *allosyndetic* pairing. Meiotic and mitotic chromosomes of the *O. australiensis* and *O. sativa*, in the hybrid were discriminated by GISH for the first time. Results showed that both genomes were highly divergent.

Key words: Genomic *in situ* hybridization, wide hybrid, chromosome pairing.

INTRODUCTION

Wide hybridization is one of the plant breeding approaches for incorporating alien genetic variation to cultivated species. The first step in incorporating desirable alien chromosome segment is to produce the hybrid between wild and cultivated species and to establish alien addition lines and introgression lines. Meiosis in wide hybrid is known to be highly irregular. This irregularity is the main

reason for sterility. Pairing failure within hybrid may be due to insufficient homology between different parental genomes. In recent breeding, however, some fertility restoration can occur and this allows subsequent back-crossing eventually leading to gene introgression from wild species into crops or as results of spontaneous chromosome doubling. Many important characteristics have been incorporated into rice by alien gene transfer for example, grassy stunt resistance from *Oryza nivara* (Khush, 1977), bacterial blight (BB) resistance (*Xa21*) from *Oryza longistaminata* (Khush et al. 1990), brown plant hopper (BPH) and white backed plant hopper (WBPH) resistance from *Oryza officinalis* (Jena and Khush, 1990), blast and bacterial blight resistance from *Oryza minuta* (Amante et al. 1992), cytoplasmic male sterility from *Oryza perennis* (Dalmacio et al., 1995) and

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Abbreviations: GISH, genomic *in situ* hybridization; BB, bacterial blight; BPH, brown plant hopper; WBPH, white backed plant hopper; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate, F1, first filial.

bacterial blight resistance from *Oryza brachyantha* (Brar et al., 1996). *Oryza australiensis* ($2n = 24$) is a diploid species having EE genome (Li et al., 1963). It is resistant to BB and BPH and is drought tolerant. It is distributed in Australia. The information on the assessment of genomic relationship between these two species by multiple approaches is lacking. The present investigation showed the genomic relationship between the chromosomes of these two species.

MATERIALS AND METHODS

Preparation of genomic DNA

The genomic DNA was isolated from 5 - 10 g fresh leaves from *O. australiensis* and *O. sativa*, using the method of Dellaporta et al. (1983). The DNA was digested with EcoR1 and labeled with Biotin-14-dATP, by nick translation (Gibco BRL), according to standard nick translation labeling system.

Genomic *in situ* hybridization

The hybridization mixture, containing 120 ng of biotinylated probe, 50% formamide, 3 µg SSS DNA, 2xSSC and 2.4 µg unlabeled *O. sativa* DNA was denatured at 80°C for 10 min and immediately quenched in ice for 5 min. An aliquot of 18 µl was dropped on each slide and covered with cover slip, sealed with paper bond and air dried. The chromosomes denatured at 80°C for 10 min, using thermal cycler (Hybaid), followed by incubation at 37°C for 18 h. The cover slips were removed in 2xSSC and the slides were washed with 2xSSC two times and once with 4xSSC at 42°C for 10 min each. An aliquot of 100 µl of blocking solution, containing 5% bovine serum albumin (BSA) in 4xSSC (4xSSC + 0.05% Tween-20), was dropped on each slide, covered with cover slip and incubated for 5 min at 37°C. An aliquot of 70 µl, fluorescein isothiocyanate (FITC)-Avidin (Boehringer Mannheim), in 1% BSA/4xSSC was layered on the slides and incubated for 60 min at 37°C. The slides were washed three times with BT buffer (Sodium carbonate + tween 20) for 10 min each at 37°C. After washing, the blocking was carried out by 5% (v/v) goat serum (Cosmo Bio. Ltd.) for 5 min at 37°C. An aliquot of 70 µl biotinylated-anti-avidin solution in 1% BSA was dropped on each slide and incubated for 60 min at 37°C. The slides were washed thoroughly with BT buffer twice and once with 2xSSC for 10 min each at 37°C, dehydrated in ethanol series: 70, 95 and 100% for 3 min each at room temperature.

The chromosomes were counterstained with propidium iodide (Sigma), 1 µl/ml in water for 2 min. Each slide was mounted with 15 µl of vectashield. The slides were screened with fluorescence microscope (Axiophot Zeiss), equipped with filter set no. 05, 09 and 25. Photographs were taken with Kodak Ektacolor, ASA/ISO 400.

Meiotic chromosome preparation

Panicles were collected from the field grown plants of *O. sativa*, *O. australiensis* and their F1 hybrid (*O. sativa* x *O. australiensis*). These were fixed in acetic alcohol (3:1) for 24 h. Anthers at suitable stages were squashed in 2% aceto-carmine under cover slip and examined for chromosome association.

Mitotic chromosome preparation

Newly emerged roots (1 - 2 cm) from field grown F1 hybrid plants

were excised and treated at room temperature with 5 mM 8-hydroxyquinoline (Sigma) for 30 min. The roots were washed thoroughly with distilled water and fixed in ethanol/glacial acetic acid (3:1) for 24 h at room temperature. To prepare chromosome squashes, the roots were taken out of fixative and thoroughly washed with distilled water and citrate buffer (0.01M Citric acid monohydrate + 0.01 M Trisodium citrate dihydrate, pH 4.6). Meristematic portion of root tips were subjected to enzymatic maceration, 3% cellulase (Onozuka R10) +2% pectolyase (Y-23) at 37°C for 1 hour. After enzyme treatment, roots were again thoroughly washed in citrate buffer and distilled water. The cells were spread on the slide in a drop of fixative (3 parts of 95% ethanol + 1 part of acetic acid). The slides were air dried and used for *in situ* hybridization.

Pollen sterility

Pollen sterility was determined from 500 pollen grains from parents and F1 hybrid. The spikelets were collected near anthesis in I₂KI solution. Darkly stained and round pollen grains were counted as fertile while lightly stained and shriveled were counted as sterile. Pollen sterility was determined as shown below.

$$\text{Pollen sterility (\%)} = \frac{\text{Sterile pollen grain(no)}}{\text{Total pollen grain (no)}} \times 100$$

RESULTS AND DISCUSSION

Three strategies were used to assess genomic relationship between *O. australiensis* and *O. sativa*: meiotic behavior in the hybrid, pollen sterility and the level of cross hybridization of genomic DNA of one species with that of the chromosomes of other species. Meiotic behavior of parents and F1 hybrid *O. sativa* x *O. australiensis* was studied at diakinesis and metaphase 1. Both the parents: *O. sativa* and *O. australiensis* showed regular meiosis with normal chromosome pairing forming 12 bivalents at diakinesis and metaphase 1. More than 95% of the cells showed normal distribution of chromosome (12/12) at anaphase 1. However in the F1 hybrid the paired chromosomes were observed at pachytene and they bifurcated at certain intervals showing structural segmental difference. There were controversial reports regarding the pairing behavior between these two species. Multani et al. (1994) observed 0-8 bivalents at diakinesis/metaphase-1 and majority of cells showed extensive pairing at pachytene. However Shastry and Rao (1961) did not observe pairing at pachytene.

This study showed limited chromosome pairing: with 0 - 4 bivalents and 16-24 univalents at diakinesis and metaphase 1 respectively (Table 1). The *O. australiensis* chromosomes were larger in size and darkly stained. In majority of PMC (55%) the chromosomes were unpaired and appeared as 24 univalent (Figure 1A). In 40% of the cells 1-2 bivalents were observed (Figure 1B) and in few cells (3.3%) 3 - 4 bivalents were recorded.

Upon detecting the nature of chromosome pairing, it was observed that the pairing may be either autosyndetic or allosyndetic (Figure 2).

All bivalents were open type. The distribution of

Table 1. Meiotic behavior of wide cross derivatives of rice.

Chromosome pairing	<i>O. sativa</i>		<i>O. australiensis</i>		<i>O. sativa</i> x <i>O. australiensis</i>	
	DK	M1	DK	M1	DK	M1
Cell analyzed (no)	75	72	151	64	98	150
Univalent						
Range	0 - 2	0 - 2	0 - 4	0 - 4	16 - 24	16 - 24
Mean/cell	0.11	0.21	0.04	2.09	22.12	22.48
Bivalent						
Range	11 - 12	9 - 12	10 - 12	10 - 12	0-4	0 - 4
Mean/cell	11.95	11.89	11.98	10.94	0.79	0.73
0II	-	-	-	-	55.00	56.00
1II	-	-	-	-	19.38	19.33
2II	-	-	-	-	19.38	19.33
3II	-	-	-	-	04.08	01.33
4II	-	-	-	-	02.04	02.00

DK = Diakinesis, M1 = Metaphase 1.

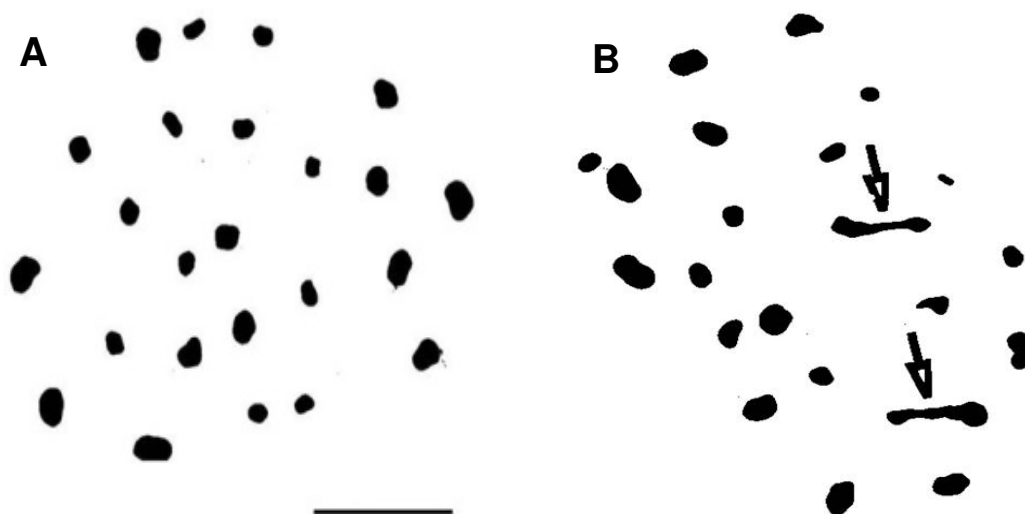


Figure 1. Meiotic chromosome behavior of F1 hybrid (*O. sativa* x *O. australiensis*). **A)** Metaphase-1 showing 24 univalent. Darkly stained and large chromosomes represent *O. australiensis* genomes. **B)** Metaphase-1 showing end to end association between chromosomes of A and E genome (arrow). Bar shows 10 μ m.

chromosomes at either pole at anaphase 1 was highly irregular. None of the cells showed regular distribution (12/12) at anaphase 1. Majority of the cells (62%) at telophase had three or more poles. Occurrence of 12 chromosomes at anaphase 1 followed by the chromosomes of the other genome at telophase were observed that clearly indicated the timing imbalance for condensation and mobility of the chromosome of these two genomes. This indicated that the meiotic cycle of one genome is much shorter than the other genome or the presence of one genome may hasten the condensation of the other genome. Limited pairing of chromosomes

indicated that the two genomes were highly divergent.

The pollen sterility is one of the indications of the level of relationship of the genomes in the hybrid. The pollen sterility was examined in F1 hybrid and compared with respective parents. Data on pollen and spikelet sterility is given in Table 2. The parents had 10 to 10.4%, pollen sterility: the hybrid was highly sterile with (99.95%) pollen sterility. The pollen and spikelet sterility may be due to irregular meiosis or unfavorable gene interaction and genomic disharmony indicating that these two genomes were divergent.

GISH is another powerful technique for assessing the

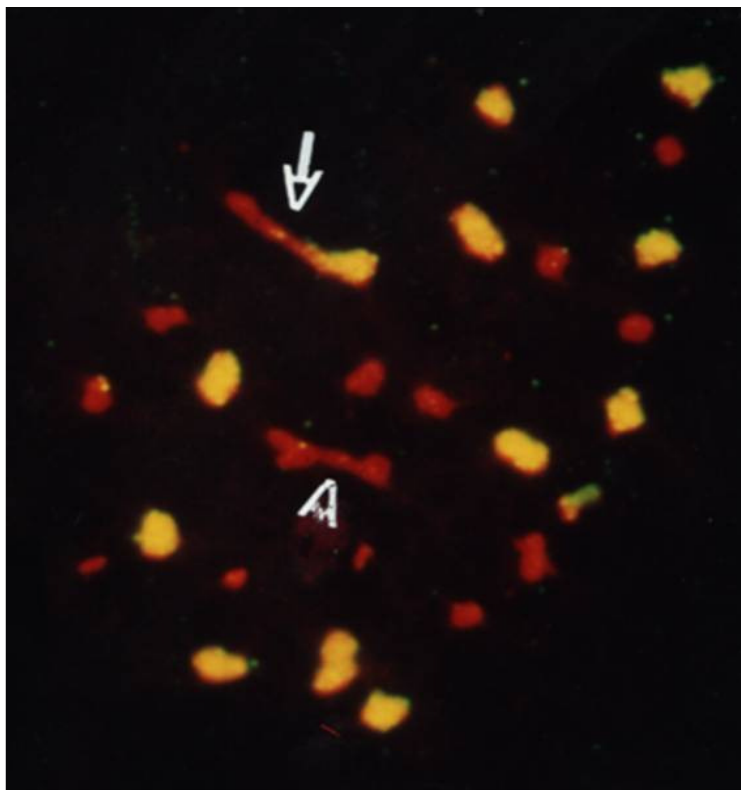


Figure 2. Differential painting of meiotic chromosomes to discriminate the chromosomes of *O. australiensis* from *O. sativa*, in hybrid. Metaphase cell showing probe hybridization to the chromosomes of *O. australiensis* which fluoresced yellow and non labeled *O. sativa* chromosomes appeared red due to counterstaining with propidium iodide. Arrow indicates the pairing between A and E genomes chromosomes and arrow head indicates the chromosome pairing within A genome chromosomes.

Table 2. Pollen and spikelet sterility in *O. sativa*, *O. australiensis* and their F1 hybrid.

Parents/Hybrid	Pollen grain			Florets		
	Stained	Unstained	Pollen sterility (%)	Filled	Unfilled	Spikelet sterility (%)
<i>O. sativa</i>	760	85	10.00	1015	135	11.7
<i>O. australiensis</i>	3840	448	10.4	400	163	28.9
<i>O. sativa</i> x <i>O. australiensis</i>	1	2111	99.9	0	700	100

genomic relationship among the species. Differential painting of chromosomes by GISH was used to look into the genomes of F1 hybrid (*O. sativa* x *O. australiensis*). The *O. australiensis* DNA when used as labeled probe to the chromosomes of hybrid as shown in Figures 3A and B, indicated that the probe preferentially hybridized to *O. australiensis* chromosomes that fluoresced green due to FITC under blue light excitation. The probe produced uniform labeling pattern over the entire length of all the *O. australiensis* chromosomes.

This indicated that the two genomes were diverse and repetitive sequences were dispersed throughout the genome. Similar results were reported by Uozu et al.

(1997) who described the chromosomes of *O. australiensis* and demonstrated that these chromosomes were larger than *O. sativa* and repetitive DNA sequences were evenly distributed on all the chromosomes. Such a labeling pattern is important particularly when using genomic DNA of wild species as a probe to detect introgression or representing the entire length of a chromosome. The *O. australiensis* chromatin was distinguishable at all the stages of the cell cycle. In interphase nuclei, *O. australiensis* chromatin appeared as distinguished domain and did not intermix with *O. sativa* chromatin. No cross hybridization of probe to the *O. sativa* chromosomes was examined indicating that two genomes were highly

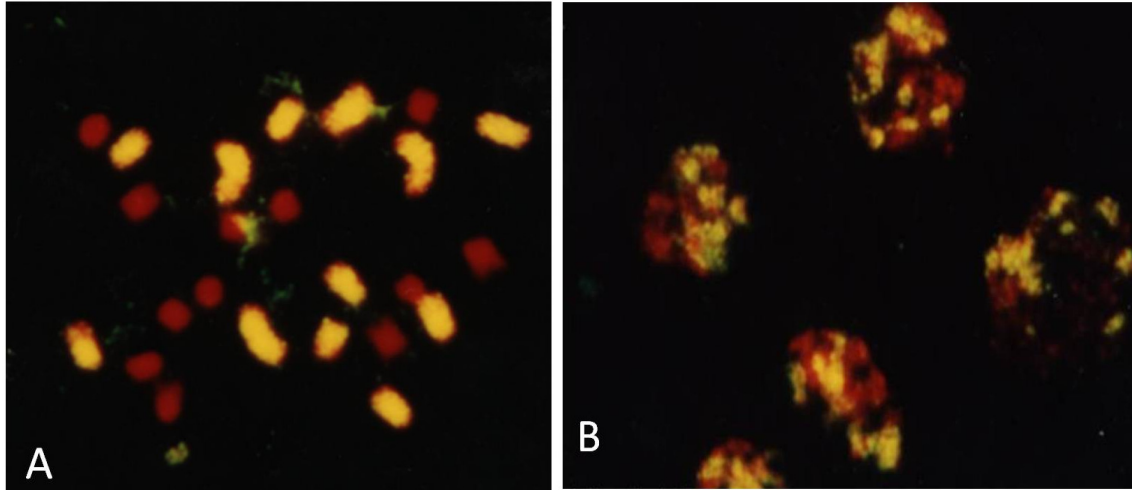


Figure 3. Genomic *in situ* hybridization using labeled *O. australiensis* DNA as probe on somatic metaphase chromosomes of the hybrid *O. sativa* x *O. australiensis*. **A)** Metaphase cell showing labeled *O. australiensis* chromosomes (yellow) and unlabeled *O. sativa* chromosomes (red). **B)** Intephase cells showing two domains of chromatin, *O. australiensis* (yellow) and *O. sativa* (red).

divergent. It was demonstrated that *O. australiensis* and *O. sativa* were highly divergent and there are rare chances of genomic reshuffling between these two species.

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