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# Identification of genome constitution of *Oryza malampuzhaensis*, *O. minuta*, and *O. punctata* by multicolor genomic in situ hybridization

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**Abstract** Multicolor genomic in situ hybridization (McGISH) was applied to identify the genomic constitution of three tetraploid species (*2n = 4x* = 48) in the *Oryza officinalis* complex of the genus *Oryza*, i.e. *Oryza malampuzhaensis*, *Oryza minuta*, and *Oryza punctata*. The genomic probes used were from three diploids, i.e. *Oryza officinalis* (CC), *Oryza eichingeri* (CC) and *Oryza punctata* (BB), respectively. The results indicated that all three tetraploids are allotetraploid with the genomic constitution of BBCC, and among them the genome constitution of *O. malampuzhaensis* was verified for the first time. Restoration of the independent taxonomic status of *O. malampuzhaensis* is suggested. One pair of satellite chromosomes belonging to the B genome was identified in *O. malampuzhaensis*, but no such satellite chromosomes were found in either *O. minuta* or the tetraploid *O. punctata*. The average chromosome length of the C genome was found to be slightly larger than that of the B-genome chromosomes of *O. minuta*, but not in the tetraploids *O. punctata* and *O. malampuzhaensis*. McGISH also revealed that the B genome of *O. minuta* and the B genome of diploid *O. punctata* showed clear differentiation from each other. Therefore, the suggestion was proposed that the B genome in diploid *O. punctata* was not the source of the B genome of *O. minuta*. The present results proved that multicolor GISH had high resolution in identifying the genomic constitution of polyploid *Oryza* species.

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## Introduction

The genus *Oryza* L., distributed in the tropics and the subtropics worldwide, is composed of 24 species, including two cultivated species, i.e. *Oryza sativa* L. and *Oryza glaberrima* Steud. *O. sativa* is an important staple food for nearly one-half of the world population. It provides 20% of the global human per capita energy and 15% of the per capita protein (Lu 1999). Wild relatives of rice are extremely valuable genetic resources that serve to broaden the genetic background of cultivated rice. A better understanding of the genomic relationships of species in the genus will facilitate effective conservation and efficient utilization of the genetic resources in *Oryza*.

To-date, six basic genome sets, AA, BB, CC, EE, FF and GG, and four genomic combinations, BBCC, CCDD, HHJJ and HHKK, have been identified and designated in diploid and tetraploid species of *Oryza*, respectively (Vaughan 1989; Aggarwal et al. 1997; Ge et al. 1999). Based on the genome constitution, Vaughan (1989) classified the species of this genus into four complexes. The *Oryza officinalis* complex (syn. *Oryza latifolia* complex, Tateoka 1962a, b) is the largest, including diploid species with BB, CC and EE genomes and tetraploid species with BBCC and CCDD genomes. *Oryza punctata* Kotechy ex Steud. is the only diploid species with a BB genome, and is confined to Africa (Katayama 1967b, 1977). At least three species, *Oryza eichingeri* A. Peter, *O. officinalis* Wall. ex Watt, and *Oryza rhizomatis* Vaughan, contain the CC genomes, and are distributed more widely (Vaughan 1989, 1994). The tetraploid species having the BC genomes are *Oryza minuta* J.S. Presl. et C.B. Presl., tetraploid *O. punctata* (Hu 1970; Kihara 1963) and tetraploid *O. eichingeri* (Kihara 1963; Tateoka 1965; Hu 1970; Lu 1998). *Oryza malampuzhaensis* Krish. et Chand. is another tetraploid species in the complex found in India

(Krishnaswamy and Chandrasekharan 1957). It was considered as a subspecies of *O. officinalis* by Tateoka (1963) and as a synonym of *O. officinalis* by Vaughan (1989). The genome constitution of *O. malampuzhaensis* was speculated to be BBCC (Krishnaswamy and Chandrasekharan 1957), but no convincing experimental evidence has been provided. Moreover, because the most-likely donor of the B genome, i.e. *O. punctata*, is confined to Africa, the genomic constitution of BBCC species which exist in Asia has been questioned (Nayar 1973; Vaughan 1989).

The genomes of the *Oryza* species were identified essentially on the basis of the observation of chromosome-pairing behavior at meiotic metaphase-I of interspecific hybrids (Morinaga 1943, 1956; Li et al. 1963; Hu 1970; Katayama 1977). This method has also provided much valuable evidence showing the differentiation between genomes (Katayama 1967a; Katayama and Ogawa 1974), or even of the same genome of different species or different ploidy levels (Katayama 1967b; Hu 1970; Katayama and Onizuka 1978). However, chromosome pairing is known to be influenced by a number of environmental and genetic factors, which in some cases has led to an inaccurate measure of genomic affinities (Gupta and Fedak 1985; Evans 1988; Kimber and Feldman 1987; Seberg 1989; Von Bothmer et al. 1989; Petersen 1991; and reviewed by Jauhar and Joppa 1996). It is, therefore, necessary to adopt more-reliable methods to assess the genomic constitution of these tetraploid wild rice species of the *O. officinalis* complex.

Genomic in situ hybridization (GISH) uses the total genomic DNA of an analyzer (a genomically known species) as a probe in order to detect the chromosomal DNA homology of the analyzer with the genome of a species with an unknown genome constitution, which provides a more direct approach for genomic studies at both chromosomal and DNA levels (Le et al. 1989; Schwarzacher et al. 1989; Jiang and Gill 1994). In situ hybridization has also been used to map genomic-specific sequences on rice chromosomes (Ohmido and Fukuii 1997). GISH was first used by Fukui et al. (1997) to identify the genomic constitution in the genus *Oryza*. Using labelled genomic DNA of *O. officinalis* (CC) as a probe, they successfully identified the chromosomes of the CC genome in *O. minuta* and *O. latifolia*. Simultaneous detection of different sequences or genomes by multicolor FISH (Schmidt and Heslop-Harrison 1996; Iwano et al. 1998) or McGISH (Shishido et al. 1998), or bicolor FISH (Pedersen and Langridge 1997), bears the advantage of recognising of different targets against the same background. Using McGISH, Shishido et al. (1998) identified the A, B and C genomes in rice somatic hybrids. The objectives of the present study are to determine the genomic constitution of the tetraploids *O. malampuzhaensis*, *O. minuta*, and *O. punctata* by an improved McGISH technology, using the total genomic DNA of *O. eichingeri*, *O. officinalis* and *O. punctata* as probes.

## Materials and methods

### Plant materials and root collection

Six wild rice species with different ploidy levels and origins were used in this study (Table 1). The diploid species were employed as analyzers, from which genomic probes were prepared; and the tetraploid species were used for preparing chromosomal spreads. All seeds were kindly supplied by the International Rice Research Institute (IRRI), except for ''Zhou-198'' which was collected in China.

Excised root tips (1–2-cm long) from vigorous plants were pretreated with 4 mM of 8-hydroxyquinoline at room temperature for 3 h and fixed in 1:3 (v/v) acetic acid: absolute ethanol for at least 24 h at 4°C, and stored in 75% ethanol at –20°C.

### Chromosome preparation

Chromosome spreads were prepared by an enzymatic-maceration and air-dry (EMA) method modified from Fukui and Iijima (1992) and Fukui et al. (1994). The fixed root tips were washed in distilled water for 10 min and root caps and matured parts were carefully removed. The dissected meristertic regions (about 1-mm long) were soaked in citric acid buffer (4 mM citric acid, 6 mM sodium citrate, pH 4.2) for 30 min, and then the materials were macerated in 4% cellulase (''Onozuka'' R-10) and 4% pectolyase (Kikkoman Co. Y-23) in citric acid buffer at 37°C for 95 min. The root tips were carefully pipetted out and thoroughly washed in the citric acid buffer, and then soaked in this buffer for 60 min. A precooled wet slide covered with poly-L-lysine (Sigma, p8920) was placed on ice, and then a root tip was added to it and smeared with a drop of cold fixative (1:3 acetic acid:absolute ethanol at 0°C). After removal of excess liquid, the slide was air-dried.

**Table 1** Wild rice species and accessions used in this study [a]

| Species | Accession | 2n | Genomic constitution | Origin |
|---|---|---|---|---|
| *O. officinalis* | Zhou-198[b] | 24 | CC | China |
| *O. eichingeri* | IRGC 101422 [c] | 24 | CC | Uganda |
| *O. punctata* | IRGC 103896 | 24 | BB | Tanzania |
| *O. punctata* | IRGC 105137 | 48 | BBCC | Zaire |
| *O. minuta* | IRGC 101082 | 48 | BBCC | Philippines |
| *O. malampuzhaensis* | IRGC 80764 | 48 | Unknown | India |

[a] All the living materials are maintained in the greenhouse of the Beijing Institute of Botany, Chinese Academy of Sciences, Beijing, China
[b] Collected in Guangxi Province, China, in 1994
[c] IRGC: International Rice Germplasm Collection stored at the International Rice Genebank, IRRI

Probe labeling

Total genomic DNA from *O. officinalis* (CC), *O. punctata* (BB) and *O. eichingeri* (CC) was extracted and labelled by nick translation with bio-14-dATP (GIBCO BRL Cat. No. 19524–016) and DIG-11-dUTP (Boehringer Mannheim, Cat. No.1093088), respectively, following the manufacturer's instruction. The labelled probes were purified with GFX PCR DNA and a Gel-Band Purification Kit (Pharmacia, 27-9602-01). The length of the probe was estimated by gel electrophoresis to be between 200 and 600 bp.

In situ hybridization and probe detection

The method of in situ hybridization and probe detection followed Leitch et al. (1994) with some modification. Chromosome spreads were treated with 100 µg/ml of DNase-free RNase in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) at 37°C for 1 h, followed by two 5-min washes in 2 × SSC at room temperature. The slides were then treated with 1µg/ml of Proteinase K at 37°C for 10 min and washed three-times in Proteinase K stop buffer (20 mM Tris-HCl, pH 8.0; 2 mM $CaCl_2$; and 50 mM $MgCl_2$) for 5-min each. The slides were further treated with 20% acetamide in 2 × SSC at 50°C for 10 minutes. After three 5-min washes in 2 × SSC at room temperature, the slides were post-fixed in 4% (w/v) paraformaldehyde at 37°C for about 10 min, washed twice in 2 × SSC for 5 min, dehydrated through a graded ethanol series (70%, 90% and 100%), and air-dried.

The hybridization mixture consisted of 50% de_ionized formamide, 2 × SSC, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS, 0.25 µg/µl of sheared salmon sperm DNA (about 100 bp), and 3–5 ng/µl of labelled probes for each slide. The probe compositions are shown in Table 2. After being denatured in boiling water for 10 min, the mixture was immediately chilled on ice for 10 min, added to the slides (40 µl for each slide), and covered with a plastic cover slip. The slides were placed in a humidity chamber and denatured at 80°C for 10 min in a hybridization oven. Hybridization was carried out in an incubator for 40 h at 37°C with gentle shaking. The slides were then immersed in 2 × SSC at room temperature to float the cover slips off, followed by the following washes at 42°C for 10 min: 50% formamide in 0.1 × SSC for *O. minuta*, 20% formamide in 0.1 × SSC for *O. punctata*, and 20% formamide in 2 × SSC for *O. malampuzhaensis*, followed by washing twice in 2 × SSC at 37°C for 5 min, and 2 × SSC at room temperature for 5 min. The stringency and post-hybridization stringency (Table 2) was calculated by using the equation described by Meinkith and Wahl (1984).

The slides were immersed in 4 × SSC containing 0.2% Tween-20 for 10 min, followed by a blocking treatment with 5% bovine serum albumin (BSA) in 4 × SSC/Tween-20 at 37°C for 30 min. Detection of a biotin-labelled probe was achieved by using avidin-FITC (Boehringer Mannheim) and a digoxigenin-labelled probe by using anti-digoxigenin rhodamine conjugation (Boehringer Mannheim). The blocking solution was drained off and 50 µl of antibody solution (5 µg/ml for each antibody in 5% BSA) was applied to each slide for 1 h at 37°C, and then the slides were washed in 4 × SSC/Tween-20 three-times (twice at 37°C for 5 min, once at room temperature for 10 min). They were then counterstained with 100 µl of DAPI (5 µg/µl) in McIlvaine's buffer (0.018 M citric acid, 0.164 M $Na_2HPO_4$) at 37°C for 20 min, followed by a brief washing in 4 × SSC/Tween-20, and mounted with one drop of FluoroGuard antifade reagent (BIO-RAD).

The chromosomes were observed with a fluorescence microscope (Leica, DMRBE). The E4-, H3- and D-excitation filters were used for the examination of the FITC, rhodamine and DAPI signals respectively. Photographs were taken with a Kodak Ektachrome 400 film. on a slide with different fluorescence signals, two or three filters were used one after another for taking a combined image. The films were scanned with a computer by a scanner (NuScan *III* Scanner, N-TEK) combined with a transparent media adapter (FilmScan 45, N-TEK). The scanned images were adjusted slightly with image-processing software (Photoshop, Adobe Systems Inc.) to enhance contrast. The chromosome relative lengths were measured by the Measure-Tool of PhotoShop. The images were printed out with a Digital color printer (P-300E, Olympus).

## Results

Genome constitution

O. punctata.

Figures 1a–d show the GISH results for the tetraploid *O. punctata* using probes from digoxigenin-labelled genomic DNA from diploid *O. eichingeri* (CC) and biotin-labelled genomic DNA from diploid *O. punctata* (BB). The hybridization stringency was 65–75%, and the washing stringency was 82–87%. Under such conditions, the hybridization signals were highly specific. Figure 1a shows the 48 chromosomes counterstained with DAPI, confirming the tetraploid nature of this species. Twenty four chromosomes showed strong bright green hybridization signals derived from the biotin-labelled B-genome probe (Fig. 1b). In contrast, the other 24 chromosomes showed strong red signals derived from a digoxigenin-labelled C-genome probe (Fig. 1c). Thus, B and C genomes were clearly discriminated on the same

**Table 2** Targets, probes, and hybridization and washing stringency

| Target | Probe and block | Hybridization stringency[a] | Washing stringency[a] |
|---|---|---|---|
| *O. minuta* (BBCC) | DIG-C[b] (*O. eichingeri* )<br>BIO-B[c] (*O. punctata* ) | 65–75% | 90–99% |
| *O. punctata* (BBCC) | DIG-C (*O. eichingeri*)<br>BIO-B (*O. punctata*) | 65–75% | 82–87% |
| *O. malampuzhaensis (4x)* | DIG-B (*O. punctata*)<br>BIO-C (*O. officinalis*) | 65–75% | 60–63% |
| *O. minuta* (BBC C) | DIG-B(*O. punctata*)<br>Block DNA (*O. officinalis*[d]) | 65–75% | 82–87% |

[a] The stringency was calculated by the equation described by Meinkoth and Wahl (1984)
[b] DIG-C: the total DNA of the C genome labelled with digoxigenin
[c] BIO-B: the total DNA of the B genome labelled with biotin
[d] Extracted from the *O. officinalis* diploid (*2n = 2x*), containing only the C genome

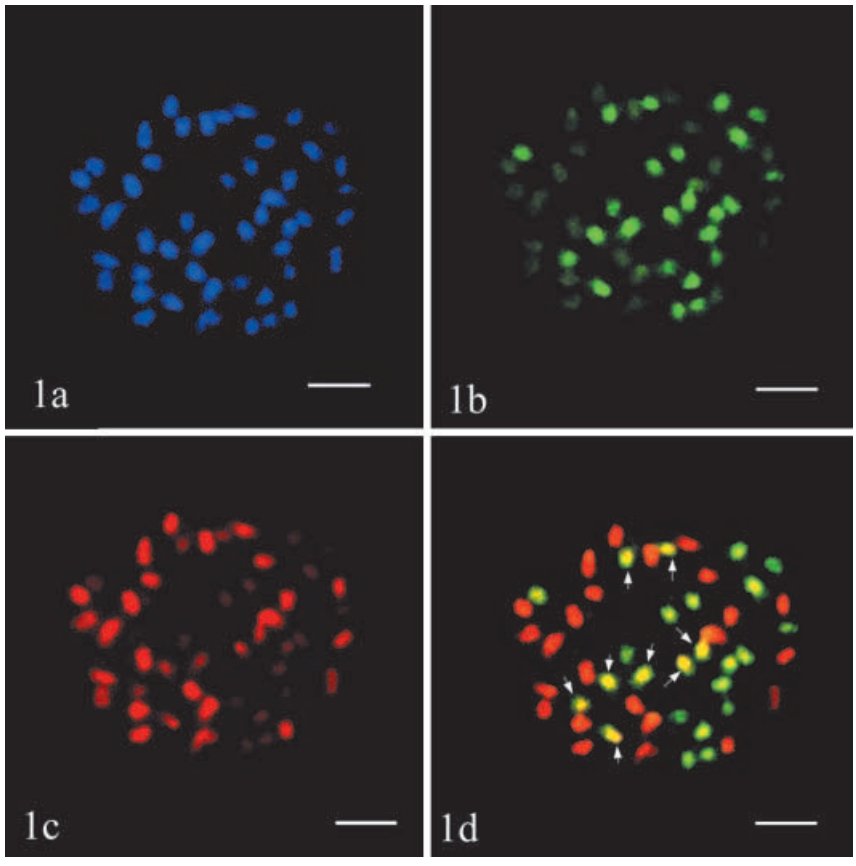


**Fig. 1a–d** GISH images of tetraploid *O. punctata* using labelled B- and C-genomic DNAs as probes simultaneously. *Bar* = 5 μm. **a** Chromosomes counterstained by DAPI. **b** The B-genome chromosomes showing strong green signals, probed by the biotinylated B-genomic DNA from diploid *O. punctata*, and detected with FITC. **c** The C-genome chromosomes showing strong red signals, probed by the digoxigenin-labelled C-genomic DNA from diploid *O. eichingeri*, and detected with rhodamine. **d** A multi-color GISH image, showing the B and C genomes clearly distinct. *Arrows* indicate the shared sites by both B and C genomes

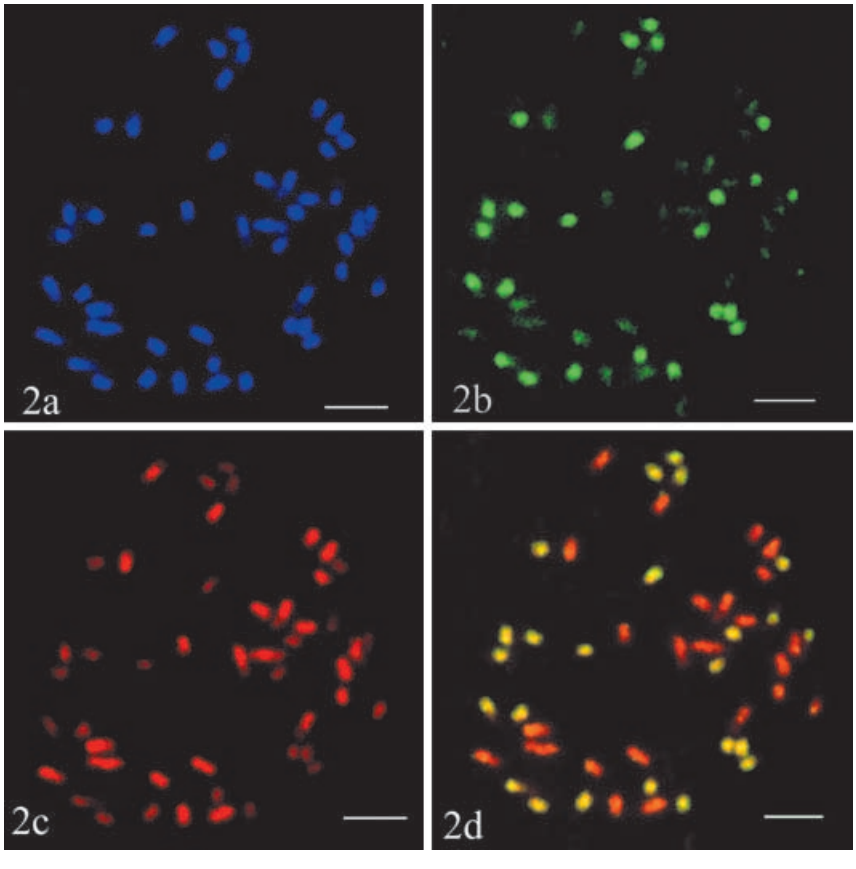


**Fig. 2a–d** Metaphase GISH chromosomal images of *O. minuta* using labelled B- and C-genomic DNAs as probes simultaneously. *Bar* = 5 μm. **a** Chromosomes counterstained by DAPI. **b** The B-genome chromosomes showing strong green signals, probed by biotinylated B-genomic DNA from diploid *O. punctata*, and detected with FITC. **c** The C-genome chromosomes showing strong red signals, probed by digoxigenin-labelled C-genomic DNA from diploid *O. eichingeri*, and detected with rhodamine. **d** A multi-color GISH image, showing the B and C genomes clearly discriminated

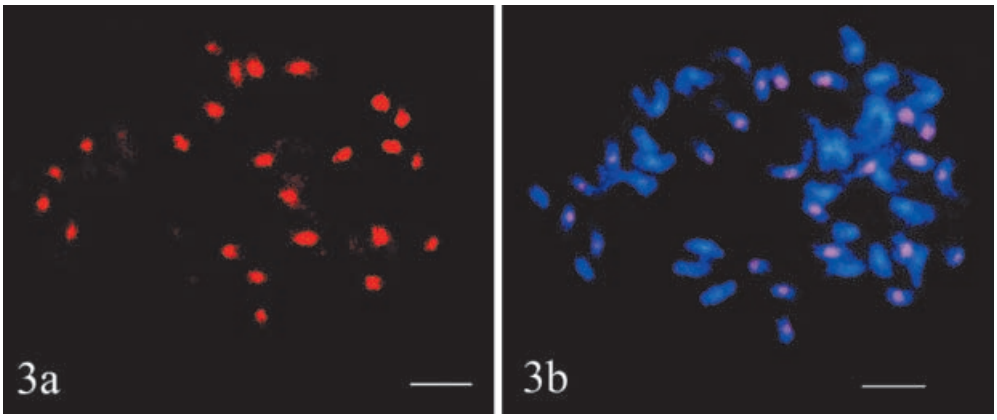


**Fig. 3a–b** Pre-metaphase chromosomal GISH images of *O. minuta* using digoxigenin-labelled B-genomic DNA from diploid *O. punctata* as a probe and unlabelled C-genomic DNA from *O. officinalis* as a block. *Bar* = 5μm. **a** Hybridization signals (red) can only be found on B-genome chromosomes. **b** The chromosomes of the B genome are clearly discriminated from those of the C genome after DAPI counterstaining (*blue*). Note that the hybridization signals only cover limited regions of the chromosomes

chromosome preparation. Several specific hybridization sites, which show overlapping color (yellowish-green) indicating co-hybridization signals of both B- and C-genome probes, were localized in the chromosomes of the B genome (Fig. 1d). These results confirmed the genome constitution of *O. punctata* to be BBCC.

O. minuta.

Figures 2a–d show the GISH results for *O. minuta* using digoxigenin-labelled genomic DNA from diploid *O. eichingeri* (CC) and biotin-labelled genomic DNA from diploid *O. punctata* (BB). The post-hybridization washing stringency was about 90–95%, and under those conditions non-specific hybridization was removed. Therefore, the signals represented the homologous nature between the probes and the target sequences. Figure 2a shows 48 chromosomes counterstained with DAPI. The B-genome probe detected with FITC, hybridized with 24 chromosomes showing strong green hybridization signals (Fig. 2b). These chromosomes should belong to the BB genome. The C-genome probe detected with rhodamine, hybridized with the other 24 chromosomes showing bright-red fluorescence signals (Fig. 2c). These should belong to the CC genome. The combined multicolor GISH image (generated by B- and C-genomic probes together) showed red and yellow signals on the same chromosome preparation (Fig. 2d). The results indicated that *O. minuta* was an allotetraploid with clearly distinct BBCC genomes. Similar results were reported by Fukuii et al. (1997).

Figure 3a, b is a prometaphase of *O. minuta* probed with digoxigenin-labelled genomic DNA from diploid *O. punctata* (BB) together with an excess of unlabelled genomic DNA from diploid *O. officinalis* (CC). Figure 3a shows that only 24 chromosomes have strong hybridizing signals while the other 24 are invisible. After counterstaining with DAPI, the hybridizing signals look violet and the non-hybridized chromosomal regions have a blue fluorescence (Fig. 3b). Most B-genome chromosomes hybridized unevenly with the probe, suggesting that the B genome of *O. punctata* and the B genome of *O. minuta* differentiated considerably for each other.

O. malampuzhaensis.

Figures 4a–d show the GISH results for of metaphase chromosomes of the tetraploid *O. malampuzhaensis* with two probes labelled by the genomic DNAs of *O. punctata* (B genome, indicated by red color) and *O. officinalis* (C genome, green color). Figure 4a shows fluorescence metaphase chromosomes counterstained with DAPI. When hybridized with the C-genome probe, 24 chromosomes showing bright green signals should belong to the C genome, while the other chromosomes gave weak signals (Fig. 4b). When probed with digoxiginen-labelled B-genomic DNA, the other 24 chromosomes show strong red signals, suggesting that they belong to the B genome (Fig. 4c). In contrast, the C-genome chromosomes showed weak signals (Fig. 4c). In the multicolor GISH image of the same chromosome preparation, 24 chromosomes with a red signal belong to the B genome and the other 24 with yellowish-green signals belong to the C genome (Fig. 4d). These results indicate that *O. malampuzhaensis* is an BBCC allopolyploid having a BBCC composition. In this experiment, the hybridization stringency was 65–75%, and the washing stringency was 60%–63% (Table 2). Under these conditions, the probe could hybridize to the target DNA with an homology of over 75%. Since the CC and BB genomes could be clearly identified at the lower stringency (65–75%), there must be considerable differentiation between the B and C genomes, although low stringency may cause some non-specific hybridization (Fig. 1c).

GISH-detected karyotypic features

Karyotypic differences were observed among the three tetraploid rice species.

(1) In the tetraploid *O. malampuzhaensis*, a pair of satellite chromosomes was identified in the B genome (Fig. 4c and d, indicated by arrows), but no B-genome chromosome satellites were found in the tetraploids *O. minuta* and *O. punctata*.
(2) In *O. punctata*, several hybridization sites showing overlapping colors (yellowish-green) were located on

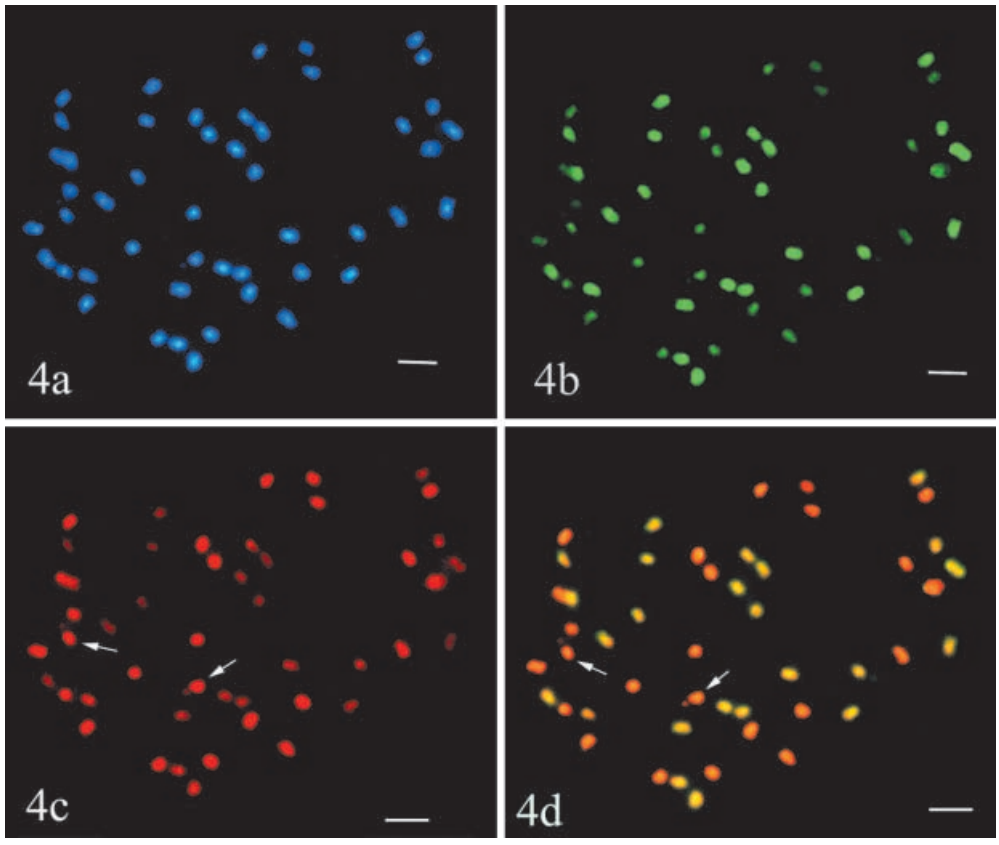


**Fig. 4a–d** GISH images of tetraploid *O. malampuzhaensis* using labelled B- and C-genomic DNAs as probes simultaneously. *Bar* = 5µm. **a** Chromosomes counterstained with DAPI. **b** The B-genome chromosomes showing strong green signals, probed by the biotinylated B-genomic DNA from diploid *O. officinalis*, and detected with FITC. **c** The C-genome chromosomes showing strong red signals, probed by digoxigenin-labelled C-genomic DNA from diploid *O. punctata*, and detected with rhodamine. *Arrows* indicate a pair of satellite chromosomes. **d** Multicolor GISH images, showing the B genome as distinct from the C genome. *Arrows* indicate the satellited chromosomes

the chromosomes of the B genome (Fig. 2d), suggesting co-hybridization of both the B- and C-genome probes.

(3) Differentiation of the B genome of *O. minuta* from that of diploid *O. punctata* was observed, as shown in Fig. 3b, indicating that the B-genomic probe of *O. punctata* did not cover the entire B-genome chromosomes of *O. minuta* but only some sites along the chromosomes.

(4) Nandi (1936, 1938) discerned 12 larger and 12 smaller bivalents in *O. minuta*; thus he considered *O. minuta* would be a natural hybrid between species with a clear differentiation in chromosome sizes, but did not give a proper explanation. In the present study, GISH images clearly show that the difference in chromosome size between the B and C genomes was obvious in *O. minuta*, as the C-genome chromosomes are larger than the B-genome chromosomes (Fig. 2d). But this was not observed in the tetraploids *O. punctata* (Fig. 1d) and *O. malampuzhaensis*(Fig. 4d). Two metaphases were measured for each BBCC species, the ratio of the total chromosome lengths of the BB to CC genomes is about 1 : 1.5 in *O. minuta*, 1: 0.9 in *O. punctata*, and 1 : 1 in *O. malampuzhaensis*.

## Discussion

The general plant morphology of *O. officinalis* and *O. malampuzhaensis* is very similar. *O. malampuzhaensis* was treated as a subspecies of *O. officinalis* by Tateoka (1963) and as a synonym of *O. officinalis* by Vaughan (1989). However, the panicle and spikelet of the two species are obviously different in respect of the following characters:

*O. officinalis*; panicle wide open, basal panicle branches whorled with spikelets densely inserted half way or more from the base of branches, pedicels 0.5–1-mm long, spikelets ovate, usually shorter than 4.5 mm and wider than 2 mm.
*O. malampuzhaensis*; panicle medium-open to open, basal branches not whorled with spikelets relatively loosely inserted from the base of branches, pedicels 1.5–2.5-mm long, spikelets oblong, usually longer than 5.5 mm and wider than 2 mm.

The fact that *O. malampuzhaensis* is an allotetraploid with BBCC genomes, while *O. officinalis* is a diploid with CC genomes, combined with the morphological evidence that these two species are different, particularly in panicle characters, leads to the conclusion that the conspecific treatment of the two species (Tateoka 1963; Vaughan 1989) is unacceptable, and restoration of *O. malampuzhaensis* as a separate species (syn. Krishnaswamy and Chandrasekharan 1957) should be considered.

Chromosome-pairing data indicated that the genomic constitutions of *O. minuta* and tetraploid *O. punctata* were BBCC (Morinaga 1943, 1956; Hu and Chang

1967). Cytogenetic evidence also showed that the differentiation between the B and C genomes of BBCC species was considerable. In the hybrid of *O. eichingri* (CC) × *O. punctata* (BB), Hu (1970) observed that the mean number of bivalents was 2.23, while Ranganadhacharyulu and Yesoda Raj (1974) reported a maximum of six bivalents with a mean of 1.6. Similar results were obtained in the hybrid of *O. punctata* (BB) × *O. officinalis* (Katayama and Ogawa 1974; Ogama and Katayama 1974). In the present study, B-genome and C-genome probes correspondingly hybridized onto the chromosomes of the B and C genomes, and co-hybridization was rare, indicating that the genomes of these tetraploid species were BBCC, and that the differentiation between the B and C genomes was quite large. These observations are consistent with the results reported by Fukuii et al. (1997). Thus, the chromosome paring data agreed with the GISH results.

The only extant diploid species with a BB genome (*O. punctata*) exists solely in Africa, whereas *O. minuta* is found only in Asia. In the hybrid of *O. punctata* (BB) and *O. minuta*, Hu observed a maximum of 12 bivalents with a mean of 11–11.9, suggesting a minor differentiation of the B genomes of *O. punctata* and *O. minuta*. Our results showed that the B genome of *O. minuta* was clearly differentiated from the B genome of *O. punctata* (BB). Therefore, it is possible that *O. punctata* was not the source of *O. minuta*. Wang et al. (1992) suggested that African *O. punctata* is a close relative of a diploid B-genome species now extinct in Asia.

GISH with one probe has been successfully applied to detect different genomes in wild plant species (Anamthawat-Jonsson et al. 1990; Bennett et al. 1992; Øgaard and Heslop-Harrison 1994; Chen and Armstrong 1994; Friesen et al. 1997; Fukui et al. 1997; Chen et al. 1998; Raina et al. 1998). However, this method could only detect one genome on one chromosome preparation. Multicolor GISH using two different genomic probes simultaneously, not only gives high resolution in discriminating between the chromosomes of different genomes in an allopolyploid species, but also provides an opportunity to study the degree of differentiation between the two genomes on the same cytological background. Our results showed that, even at low stringency, multicolor GISH could produce a better result (Fig. 1d) than GISH (Fig. 1c) with one probe. High stringency further enhances the power of the method.

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