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Identification of the rice D-genome chromosomes by genomic *in situ* hybridisation

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Abstract The 24 rice D-genome chromosomes were identified among the 48 chromosomes of *O. latifolia*, which comprise the C- and D-genomes, using genomic in situ hybridisation (GISH). The B-genome chromosomes were also discriminated from the C-genome chromosomes in *O. minuta* (BBCC) by GISH. A comparison of the differences in the fluorescence intensity between the C and D genomes within *O. latifolia* (CCDD), and between the B and C genomes within *O. minuta*, indicated that the overall nucleotide-sequence homology between the B and C genomes is less than that between the C and D genomes. The origin of the D genome and the phylogenetic relationship of the D genome among the rice genomes are discussed, based on the results obtained.

Key words Genomic in situ hybridisation (GISH) · Genus *Oryza* · *O. latifolia* (CCDD) · *O. minuta* (BBCC) · Rice D genome

Introduction

Morinaga (1939) identified five different genomes, A, B, C, D and E, in the genus *Oryza* by microscopical observation of the meiotic configurations in hybrids between the different rice species. The diploid rice species *O. sativa*, *O. punctata*, *O. officinalis* and *O. australiensis* represent the A, B, C and E genomes, respectively. To-date, six different genomes are known, because another genome, F, was assigned to *O. brachyantha* (Li et al. 1961). Since then no additional genome has been reported in the genus *Oryza* for more than 35 years.

The D genome has been found only in tetraploid rice species ($2n = 4x = 48$) in combination with the C genome as CCDD. Three tetraploid species with a CCDD genome, *O. latifolia*, *O. alta* and *O. grandiglumis*, are distributed in Central and South America (Vaughan 1989). No diploid rice species in these regions has been classified as a D-genome species, and two diploid species, *O. officinalis* and *O. eichingeri*, with a C genome have been identified in Asia and Africa (Vaughan 1989).

Not only has no diploid D-genome species been found in Central and South America, but none has been found anywhere yet in spite of a world-wide search and efforts to identify a D-genome species. There have been many views on possible diploid rice species with a D-genome, the most common explanations being that the D genome has already become extinct and remains only in the tetraploid CCDD species (Jena and Kochert 1991), that the D genome is merely a variant of the C genome (Bao-Rong Lu, personal communication), or that it may have simply not yet been found.

Thus, the discovery and identification of a rice D-genome diploid species has been one of the key issues in the genetic, evolutionary and phylogenetic studies of the genus *Oryza*. The rice CCDD genome is not only important for its basic biological aspects, but also has economic importance as a breeding material containing genes for resistance to brown plant hopper (Heinrichs et al. 1985) and bacterial blight, and for a vigorous growth habit (D.S. Brar, personal communication), to which the D genome should contribute.

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Recently, development of an in situ hybridisation (ISH) method made it possible to detect repeated sequences even in rice, which has very small chromosomes compared with the other grass species in which ISH has been extensively used (Fukui 1990; Iijima et al. 1991; Fukui et al. 1994b; Ohmido and Fukui 1995). Retrotransposable elements and genome-specific repeated sequences have also been localized successfully on rice chromosomes (Uozu et al. 1997; Ohmido and Fukui 1997). Genomic in situ hybridisation (GISH), which uses total genomic DNA as the probe, is also an effective method for molecular cytogenetic studies in plants (Heslop-Harrison et al. 1990; Mukai and Gill 1991; Schwarzacher et al. 1989; 1992).

In the present paper, we report the successful identification of 24 rice D-genome chromosomes in a tetraploid species (*O. latifolia*, with 48 chromosomes belonging to the C and D genomes) on a glass slide using GISH in conjunction with an imaging method. The phylogenetic relationship of the D genome among rice genomes is also discussed, together with the result of GISH on another tetraploid species with a BBCC genome, *O. minuta*.

Materials and methods

Plant materials and cytology

Two tetraploid (amphidiploid) wild rice species, *O. minuta* Presl. ($2n = 4x = 48$, BBCC) and *O. latifolia* Desv. ($2n = 4x = 48$, CCDD), were used for the in situ identification of their chromosomes. *O. officinalis* Wall ($2n = 2x = 24$, CC) was employed to extract C-genome-specific total DNA. Table 1 shows the sources of the three wild rice species.

The root tips, 1–2 cm long, were excised and fixed in an ethanol : acetic acid (3 : 1) fixative after pre-treatment with 2 mM of 8-hydroxyquinoline solution for 4 h at room temperature. Chromosome samples were prepared using an enzymatic maceration/air-drying (EMA) method (Fukui 1996; Fukui and Iijima 1991, 1992). Good chromosome spreads which retained all 48 chromosomes and which had no overlapping chromosomes were selected for the GISH experiments.

Genomic in situ hybridisation (GISH)

Total DNA of a diploid rice species, *O. officinalis*, with a C genome, was extracted by the CTAB method (Murray and Thompson 1980), and was biotin-labelled by a random primer labelling method according to the supplier's instructions (Nippon Gene Co., Ltd., Tokyo, Japan). A hybridisation mixture (100 ng of labelled probe/slide dissolved in a mixture of equal parts of 50% formamide and 2 × SSC) was denatured for 10 min at 100°C, and immediately cooled down to 0°C. A series of post-treatments, such as treatments with an enzyme mixture (2% Cellulase Onozuka RS, 1.5% Macerozyme R-200, 0.3% Pectolyase Y-23), Proteinase K (1 mg/ml), 45% acetic acid, or RNase A (1 mg/ml) prior to GISH were applied according to Fukui et al. (1994 b).

A 17-µl aliquot of the hybridisation mixture was layered onto each glass slide, covered with a coverslip, sealed with liquid gum Arabic and then air-dried. The glass slides with a sealed coverslip were placed on a modified thermal cycler with a flat aluminium plate (Fukui et al. 1994 b; PHC-3, Techne, Cambridge, UK) at 70°C for 6 min, and then at 37°C overnight. After molecular hybridization the glass slides were washed once with 2 × SSC, twice with 50% formamide/2 × SSC, and once each with 2 × SSC and 4 × SSC at 40°C for 10 min each. Then, 5% BSA in BT buffer (BT buffer consists of 0.1 M sodium hydrogen carbonate with 0.05% Tween-20, pH 8.3) was layered onto the samples and incubated for 5 min at 38°C. Following this a 70-µl aliquot of fluorescein isothiocyanate (FITC)-avidin (0.5%, Boehringer Mannheim) in 1% bovine serum albumin (BSA) in 4 × SSC was dropped onto the glass slides, which were incubated at 38°C for 3 h. After washing the FITC-avidin solution with BT buffer three times at 40°C for 10 min each, 70 µl of a biotinylated anti-avidin solution (0.05% in BT buffer, Vector Laboratories, Calif., USA) was layered on the glass slides at 38°C for 3 h. After washing three times with BT buffer at 40°C for 5 min each, blocking with 5% BSA was carried out twice at 38°C for 5 min. Then 70 µl of a fluorescein-avidin solution (0.05%, Vector Laboratories) was layered again onto each slide. The slides were incubated at 38°C for 3 h followed by washing twice with BT buffer and once with 2 × SSC, each at 40°C for 10 min. The FITC signals were enhanced by a secondary immunological reaction with biotinylated anti-avidin as described above. Finally, the chromosomes were counterstained with propidium iodide (PI, 12.5 µg/ml) and were observed with a fluorescence microscope (Axiophot, Zeiss).

Image analyses of GISH images

A color CCD camera (HCC-3600P, Floubel, Tokyo) was used for direct digital capture of the fluorescent signals, both from the probe DNAs and from the counterstained chromosomes. The digitized fluorescence images were stored in a chromosome image-analyzing system II (CHIAS II: Fukui 1986; Fukui and Nakayama 1996; Nakayama and Fukui 1997), whose main frame is a universal image analyzer (VIDAS/IBAS, Kontron, Germany).

The signal images were analyzed by constructing an original program for the analysis of GISH images. Briefly, yellow (FITC) fluorescence signals visualized with UV exciting light were captured and stored in an image-frame memory with a 512 × 512 pixel matrix size (8-bit gray levels for each pixel). The signal image was digitally enhanced by a normalisation filter (threshold value = 1) and subjected to smoothing by the application of a median filter (3 × 3 pixel matrix). Threshold grey values were determined for each of the images so that the 24 labelled C-genome rice chromosomes could be

Table 1 Origin of the rice accessions

Species	Accession number	Chromosome number	Genome	Distribution	Source[a]
O. minuta	101125	2n = 48	BBCC	Southeast Asia	IRRI
O. latifolia	Ya-9	2n = 48	CCDD	South America	NARC
O. officinalis	W0002	2n = 24	CC	Southeast Asia	NIG

[a] IRRI: International Rice Research Institute, The Philippines; NARC: National Agricultural Research Center, Japan; NIG: National Institute of Genetics, Japan

clearly discriminated from chromosomes belonging to another genome. In other words, the fluorescence intensity of C-genome chromosomes was used as an internal standard to determine the gray values discriminating the C genome from the other genomes. The pixels with lower gray values or weaker fluorescence signals than the threshold gray value were eliminated.

The red propidium-iodide fluorescence from chromosomes was also captured and the non-specific signals other than the 48 chromosomes were eliminated. The two digital fluorescence images of the signals and chromosomes were then combined into a composite image. A look-up table, "chromoart02", was applied to reproduce the original color GISH images by false coloration.

Results

Painting C-genome chromosomes in *O. minuta*, a BBCC species

Figure 1 shows the results of GISH for *O. minuta* with the probe derived from the labelled total DNAs of *O. officinalis* (C genome only). Figure 1 a shows the 48 *O. minuta* chromosomes counterstained with red PI fluorescence excited by green light. Application of the pre-treatments made them evenly contracted to rod- or dot-like shapes with similar morphology. Although fully contracted metaphase chromosomes are impossible to identify by their condensation patterns, i.e., the uneven condensation of small plant chromosomes appearing at the pro-metaphase stage (Fukui and Iijima 1991, 1992; Fukui 1996), they were well spread and gave stronger fluorescence signals than the prometaphase chromosomes with dispersed regions.

Figure 1 b shows the enhanced fluorescence image before the elimination of sprinkled salt-like background noise and signals with lesser intensity. The post-treatments greatly reduced cytoplasmic debris and cellular proteins and RNAs, which cause non-specific hybridization to the samples. Differences in the signal intensity were obvious among the 48 chromosomes, and it was possible to visually identify 24 chromosomes with more intense signals than the other 24 chromosomes, without further image analysis. This fact indicated that the difference in the nucleotide sequences between the B and C genomes was quite large.

Figure 1 c shows the results of thresholding the fluorescence signals at the gray value which discriminated the 24 chromosomes. The contour lines of the chromosomes which were classified as having more intense fluorescence signals were extracted. To analyze the signal distribution more precisely, the contour lines of the signals were superimposed on the black and white counterstained chromosome images. As a result, the signals were localized on 24 of the rice chromosomes, indicating clearly that those were the chromosomes belonging to the C genome and that the rest were most probably B-genome chromosomes.

Figure 1 d depicts the composite image of two fluorescent images of PI-stained chromosomes (red) and FITC signals from total DNA of the C genome (yellow-green). At the mid-metaphase stage, fluorescence signals were emitted from whole chromosomes with few exceptions. Chromosomes showing a fluorescence signal emitted from a limited region could be submedian chromosomes 4 and/or 12, where a heavily condensed region was observed at one end of the chromosome at the pro-metaphase stage (Fukui and Iijima 1991; Iijima et al. 1991).

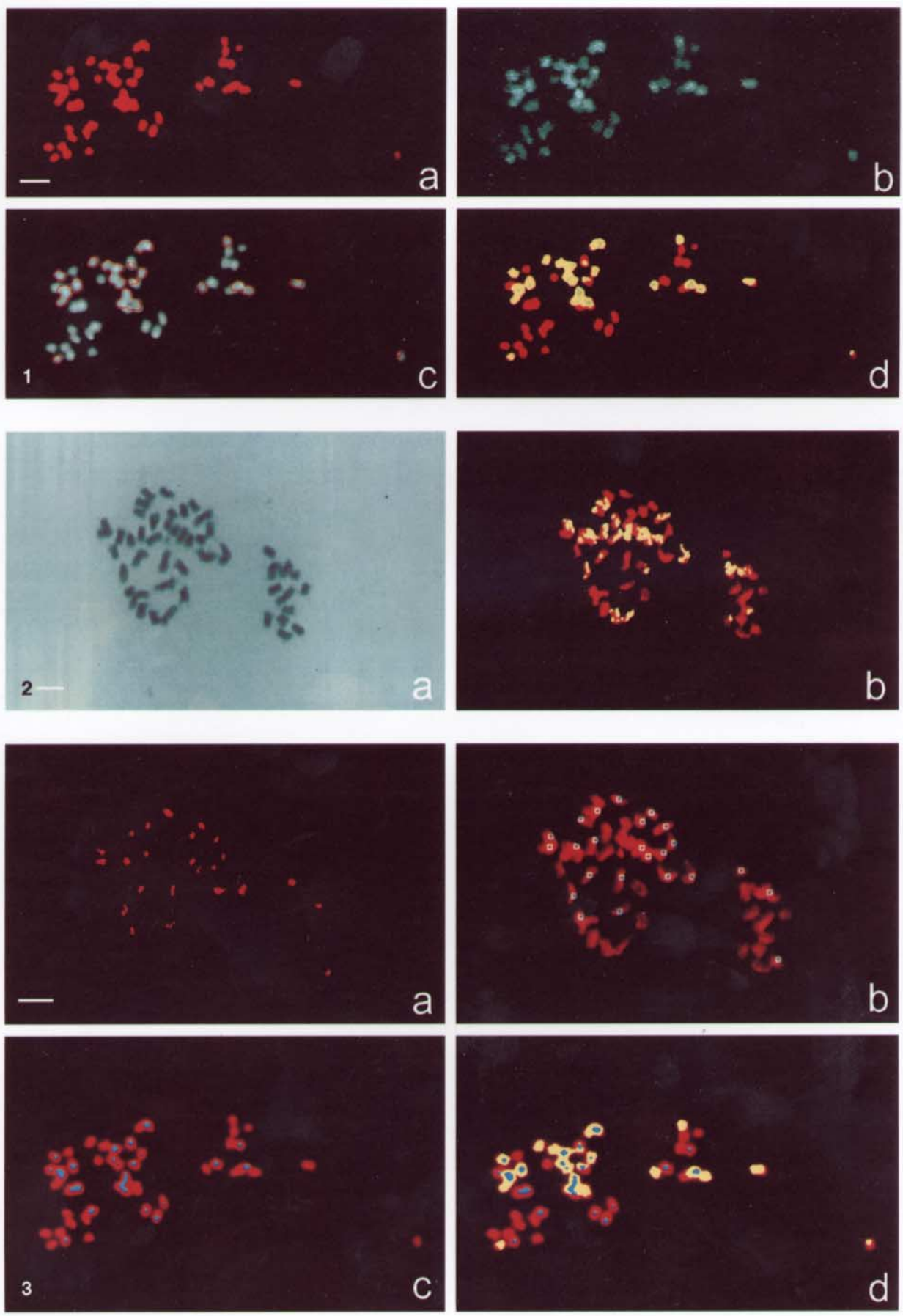
Identification of the D-genome chromosomes in *O. latifolia*, a CCDD species

Figure 2 summarizes the results of chromosome painting for *O. latifolia* with the probe derived from the total DNA of *O. officinalis*. Figure 2 a shows a Giemsa-stained chromosomal complement with 48 chromosomes belonging to either the C or the D genome. Because the chromosomes did not contract fully, identification of each chromosome was not possible, except for some characteristic chromosomes, using morphological means such as the condensation pattern (Fukui and Iijima 1991, 1992; Fukui 1996). Figure 2 b shows a composite image of the fluorescence signals emitted from the probe for total DNAs (yellow) and the PI-counterstained chromosomes (red). Twenty four chromosomes had yellow fluorescence signals, indicating that they belonged to the C genome.

The rest of the chromosomes, without fluorescence signals, were considered to be from an ancestral D-genome diploid species. These showed no significant morphological differences from C-genome chromosomes by visual inspection of the Giemsa-stained preparations. The fluorescence signals did not cover the whole of the chromosome in all cases, but showed inhomogeneous and/or banded patterns, making it difficult to discriminate them from the unlabelled chromosomes. This indicated relatively high homology in the nucleotide sequences between the C and D genomes, because the difference in signal intensity between C- and D-genome chromosomes was low and regions with intense signals from C-genome chromosomes were limited even within single chromosomes.

Intense fluorescence from centromeric regions of *O. latifolia* and *O. minuta* chromosomes

Figure 3 indicates the strong fluorescence signals observed at the centromeric regions on the chromosomes counterstained with PI for both *O. minuta* and *O. latifolia*. Figure 3 a shows the chromosomal area with the more intense signals extracted from the entire chromosomal regions of *O. latifolia*. Twenty four regions were extracted and their centers of gravity, which approximated to the centromeric positions, were superimposed on to the chromosomal images as the white


a
b
1
c
d
2
a
b
a
b
3
c
d

squares shown in Fig. 3 b. Twelve squares were distributed on the chromosomes with intense signals after GISH, and the other 12 squares were found on the chromosomes without strong signals after GISH. This clearly indicated that the intense centromeric signals after counterstaining were distributed equally on the C- and D-genome chromosomes.

The same phenomenon was evident in the case of *O. minuta* after counterstaining the chromosomes with PI. Figure 3 c shows the *O. minuta* chromosomes with the area of intense signal indicated by a blue color. Figure 3 d depicts a composite image of the GISH and of the intense centromeric signal. Half the intense signal areas were located at the centromeric areas of the chromosomes with the signals, and the other half were on the chromosomes without the signals. Thus it was also clearly demonstrated in the case of *O. minuta* that the B- and C-genome chromosomes shared equally the intense centromeric signals seen with counterstaining after GISH treatment.

Discussion

GISH using two rice amphidiploid species demonstrated the following conclusions. First, GISH is effective for discriminating the different genomes with very small chromosomes such as those of rice. Although there have been many reports on GISH using large chromosomes, such as those of *Crocus* (Ørgaard et al. 1995), wheat (Mukai 1996), etc., the results described in the present paper show the first reproducible results with GISH using small chromosomes but without blocking DNA. Since a diploid rice species with a D genome has not been found, it was not possible to apply an ordinary GISH procedure using blocking DNA to suppress random hybridisation of the probe DNA to the chromosomes. The imaging method greatly improved the selection of the labelled chromosomes by enhancing the difference of fluorescence intensity between the chromosomes belonging to the different genomes. As a result, rice B- and D-genome chromosomes were unequivocally discriminated from C-genome chromosomes, as demonstrated in Figs. 1 and 2. Second, D-genome chromosomes, which had previously been identified and observed, seemed not to be very different morphologically from C-genome chromosomes. No chromosome with specific morphological traits was observed. This fact had previously made it very difficult to identify D-genome chromosomes without using GISH.

Third, the fact that all the D-genome chromosomes could be identified using GISH makes it feasible to clone D-genome-specific DNA sequences, if GISH were to be applied prior to micro-dissection and direct-cloning methods. Fukui et al. (1991, 1992) and Kamisugi et al. (1993) have already demonstrated the methodological feasibility of eliminating specific chromosomes from chromosome samples by using laser microbeam irradiation. Successful direct cloning of certain nucleotide sequences from dissected nuclei and chromosome fragments has been reported (Fukui et al. 1994 a; Pich et al. 1994).

The phylogenetically important information that was obtained visually after GISH is the difference in fluorescence intensity between the chromosomes belonging to the different genomes within a nucleus. Thresholding the fluorescence intensity by a certain gray value to discriminate C-genome chromosomes is easy when the labelled C-genome chromosomes in general have much more intense fluorescence than the remaining B- or D-genome chromosomes. The labelled C-genome chromosomes of *O. minuta* showed much stronger fluorescence than the unlabelled B-genome chromosomes, resulting in the clear discrimination of C-genome chromosomes from B-genome chromosomes. The labelling of the C-genome chromosomes in *O. latifolia* showed inhomogeneous and/or banded labelling patterns, and hence a smaller difference in fluorescence intensity between the labelled (C-genome) and the unlabelled (D-genome) chromosomes.

Figure 4 depicts the genetic distance among the individual B, C and D genomes based on the fluorescent intensities detected between B and C, and C and D genomes. Using the C genome as a pivotal genome, the genetic distance from the B genome is larger than

Fig. 1a–d GISH of *O. minuta* (BBCC) chromosomes. **a** Chromosomes counterstained with propidium iodide. **b** Enhanced fluorescence signals after GISH treatment. **c** Counterstained chromosome images superimposed with red contour lines of the intense signal regions. **d** A composite chromosome image of counterstaining and signal images. Bar indicates 10 μm

Fig. 2a, b GISH of *O. latifolia* (CCDD) chromosomes. **a** Giemsa-stained chromosomes before GISH. **b** A composite chromosome image of counterstaining and signal images. Bar indicates 10 μm

Fig. 3a–d Intense centromeric fluorescence detected after counterstaining. **a** Extracted centromeric regions with intense fluorescent signals. **b** Superimposition of the centers of gravity of the intense signals (*white rectangles*) onto the *O. latifolia* chromosomes (*red*). **c** Counterstained chromosome images (*red*) of *O. minuta* with intense fluorescent regions as blue. **d** A composite chromosome image counterstained (*red*) with both the signal (*yellow*) and intense fluorescent region (*blue*) superimposed. Bar indicates 10 μm

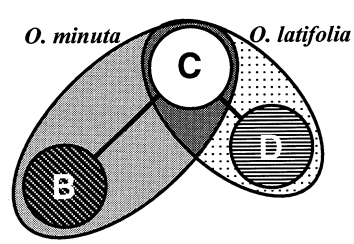


Fig. 4 Schematic representation of the genetic relationship among the three genomes. The lengths between the genomes are in arbitrary units

that from the D genome. Dally and Second (1990) compared the nucleotide sequences of chloroplast DNA for several cultivated and wild species of rice, including *O. minuta* and *O. latifolia*. They found that the sequence homology between BB and CC genomes is lower than that between CC and CCDD genomes. Although it is not possible to compare the genetic diversity of each individual genome independently by the analysis of chloroplast DNAs, the basic tendency is consistent with the results described in the present paper.

Cordesse et al. (1992) analyzed the ribosomal RNA gene spacer length in cultivated and wild rice species. They found a series of 250–260-bp repeats in the rDNA spacer regions of the AA-genome species cross-hybridized with the BB, CC and BBCC genomes, but not with the CCDD genome. This result implies that the CCDD species is more distant from the AA species than from the BB, CC and BBCC species. Jena and Kochert (1991) identified C- or D-genome-specific RFLP markers and examined the relationship between the C and D genomes. The phylogenetic trees obtained by parsimony analysis for CC- and DD-genome-specific RFLPs were similar to the combined CCDD tree, although some minor differences in the branching patterns were observed. Their result indicated that the C and D genomes are closely related. Wang et al. (1992) studied the phylogenetic relationships of species in the genus *Oryza* by using nuclear RFLPs. As a result, they estimated that the genetic distance between *O. officinalis* and *O. latifolia* is less than that between *O. officinalis* and *O. minuta*, according to the dendrogram based on mean genetic distance between the rice species. Although the result obtained by Cordesse et al. (1992) makes it rather difficult to interpret the relationship between the B, C and D genomes, there are some findings consistent with those described in this paper among previous reports involving both in the nuclear and cytoplasmic DNA analyses. Our result is not incompatible with a C-genome origin of the D genome.

The intense signals observed at the centromeric areas were sometimes seen in the chromosomes counterstained with fluorochrome after fluorescence ISH (FISH). In fact, it is known empirically that the centromeric regions were intensely stained by the counterstain after FISH in chromosomes from several plant species.

The Giemsa C-banding method was developed as a by-product during the course of development of an in situ hybridization (ISH) method (Friebe et al. 1996). Giemsa solution stains several regions of chromosomes darkly after C-banding treatment. The darkly stained regions often consist of specific repeated sequences, which was proved by FISH using repetitive sequences in wheat (Mukai et al. 1992).

In the case of rice chromosomes, no C-band-positive region has been reported to-date. This implies that another mechanism may exist to produce intense fluorescence with DNA-binding fluorochromes. The fact that only half the chromosomes had intense fluorescence suggests that the stronger fluorescence is not due to centromere-specific repeated sequences, but may be due to functional differences among the chromosomes. It is clearly indicated that the difference in fluorescence intensity is not related to the difference between the genomes to which each chromosome belongs, also suggesting a functional difference such as activation or inactivation at chromosomal and/or genomic levels, although further studies are obviously necessary to confirm this.

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