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Differentiation and inter-genomic relationships among C, E and D genomes in the *Oryza officinalis* complex (Poaceae) as revealed by multicolor genomic in situ hybridization

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Abstract The multicolor genomic in situ hybridization (McGISH) method was used to study differentiation and relationships among the C, D and E genomes in the *officinalis* complex of the genus *Oryza*. The chromosomes of *Oryza alta* (CCDD genomes) were hybridized with labelled probes of the C genome (from diploid *Oryza eichingeri* and *Oryza officinalis*) and the E genome (from *Oryza australiensis*) simultaneously. By adjusting the post-hybridization washing stringency in a gradual series, differentiation between the genomes was detected according to the homology between the target genomes and the probes. The McGISH results indicate that the C, D and E genomes share a substantial amount of similar sequences, and differentiation between the D and C genomes of *O. alta* is less than that between the E genome and each of the C and D genomes. The differentiation within the C genomes of the diploid species (*O. officinalis* and *O. eichingeri*) and the C genome of *O. alta* was clearly discerned by McGISH, suggesting strongly that neither *O. officinalis* nor *O. eichingeri* was the direct C-genome donor of *O. alta*. The evidence of the GISH results also indicates that the E genome was considerably differentiated from the C and D genomes. Therefore, the E genome should not be the direct donor of *O. alta*; on the contrary, the E genome is closer to the C than to the D genome. McGISH is an efficient method in revealing the relationships among the genomes in question, particularly under the gradual stringent-washing condition.

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Introduction

The genus *Oryza* is divided into four complexes (Vaughan 1989), and the *Oryza officinalis* complex is the largest one, comprising four different genomes B, C, D and E. The diploid species *Oryza punctata* represents the B genome, and three diploid species, *Oryza officinalis*, *Oryza rhizomatis* and *Oryza eichingeri*, have been found to have the C genome (Vaughan 1989, 1994). The only extant E-genome carrier is reported to be an Australian diploid species, *Oryza australiensis*, (Li et al. 1963). Three tetraploid species, *Oryza latifolia*, *Oryza alta* and *Oryza grandiglumis* containing the CD genomes, are geographically confined in Central and South America (Vaughan 1989). To-date, no diploid species (*2n* = 24) carrying the D genome has been reported.

Genomic in situ hybridization (GISH) was used to study relationships among the B, C and D genomes (Fukui et al. 1997), and the result suggested that the D genome was closer to the C than to the B genome. Relationships among the C, D and E genomes are currently unknown. Many attempts have been made to detect evolutionary relationships between *O. australiensis* and the CCDD species, but inconsistent results were obtained from these studies. Based on the *Adh* and *matK* gene phylogenies, Ge et al. (1999) inferred *O. australiensis* to be most closely related to the DD-genome progenitor of the CCDD species. Wang et al. (1992) concluded that *O. eichingeri* (C genome) and *O. australiensis* were the closest living relatives of the CCDD species, respectively. Evidence also suggested that *O. australiensis* was related to the CCDD species (Aswidinnoor et al. 1991; Katayama 1982; Zhao et al. 1989), but the results were insufficient to determine whether *O. australiensis* was the donor of the D genome. By contrast, some authors argued that *O. australiensis* was the least related to the

CCDD species in the *O. officinalis* complex (Ichikawa et al. 1986; Dally and Second 1990).

GISH provides an alternative tool for directly and effectively identifying the genomic constitution and detecting genome relationships (Bennett et al. 1992; Chen and Armstrong 1994; Ørgaard et al. 1994; Chen et al.1998; Raina et al.1998). Multicolor FISH techniques are an immensely powerful tool for simultaneous discrimination of each genome in allopolyploids or for mapping different DNA sequences (Schmidt and Heslop-Harrison 1996; Iwano et al. 1998; Shishido et al. 1998). The purpose of the present study is to identify the C and D genomes in the tetraploid CCDD species and to confirm the relationships among the C, D and E genomes by an improved McGISH method.

Materials and methods

The genomic DNA isolated from three diploid wild rice species, *O. officinalis* (*2n* = 24, CC), *O. eichingeri* (*2n* = 24, CC) and *O.australiensis* (*2n* = 24, EE), were used as probes. The tetraploid *O. alta* (*2n* = *4x* = 48, CCDD) was used to prepare chromosome spreads for genomic in situ hybridization. The plant materials used in the study are described in Table 1.

Chromosome spreads were prepared by an enzymatic maceration/air-drying method (Fukui and Iijma 1992; Fukui et al. 1994). Total genomic DNA from *O. officinalis*, *O. eichingeri* and *O. australiensis* was labelled respectively by nick translation with bio-14-dATP (GIBCO BRL Cat. No. 19524-016) and DIG-11-dUTP (Boehringer Mannheim, Cat. No. 1093088), following the manufacturer's instructions. The GISH method followed Leitch et al. (1994) with modifications in the stringency control of post-hybridization washing. The stringency control was undertaken by adjusting the Na^+ concentration, the temperature and the concentration of formamide in the washing solutions. The stringency values (see Table 2) were calculated according to the equation described by Meinkoth and Wahl (1984). The biotinylated-probes were detected by using avidin-FITC (Boehringer Mannheim), and the digoxigenin-labelled probes by using the anti-digoxigenin rhodamoine conjugate (Boehringer Mannheim), respectively. The chromosome spreads were counterstained with DAPI and mounted with antifade reagent (Bio-Rad). When further amplification of signals was necessary, 100 µl of 5% (v/v) normal goat serum in 4 × SSC/Tween-20 was added to each slide, and the slides were incubated for 30 min at 37°C. After that, 50 µl of monoclonal anti-avidin biotin conjugate (SIGMA, B-9655)(5 µg/ml in 5% goat serum solution) was added to each slide and incubated at 37°C for 1 h. After three washes, each for 5 min at 37°C in 4 × SSC/Tween-20, the slides were treated again with 5% BSA at 37°C for 30 min. Then another 50 µl of avidin-FITC in 5% BSA was added to each slide and incubated at 37°C for 1 h. The slides were washed in 4 × SSC/Tween-20 at 37°C three times each for 5 min, counterstained with DAPI and mounted with antifade reagent (Bio-Rad). The chromosomes were examined with a fluorescence microscope (Leica, DMRBE). Photographs were taken with Kodak Ektachrome 400 film. The images were scanned by a computer, then adjusted slightly with Adobe PhotoShop to enhance contrast, and printed out with a Digital color printer (P-300E, Olympus).

Results

Figure 1 shows McGISH images of *O. alta* (CCDD). The genomic probe from *O. officinalis* (CC) was labelled with biotin and detected with FITC (green color), while the genomic probe of *O. australiensis* (EE) was labelled with digoxigenin and detected with rhodamine (red color). The hybridization stringency was between 65 and 75%, and the post-hybridization washing stringency was between 50 and 60% (Table 2). Figure 1a shows all 48 chromosomes of *O. alta* stained by DAPI. Figure 1b shows that strong hybridization signals (green), excited by C-genomic probes, are located on every chromosome, but are unable to discriminate their identity as C or D genomes. When using the E-genomic probe, a uniform signal (red) was observed on all chromosomes (Fig. 1c). Figure 1d shows an overlapping of hybridization signals (yellow) of the C-genomic probes (green) and the E-genomic probes (red). These results indicate that these genomes share a substantial amount of similar sequences. Figure 2 shows McGISH images using the same probes as in Fig. 1, but applying a higher post-hybridization washing stringency,

Table 1 Rice species and accessions used in this study

Species	Accession no.	Chromosome number	Genome constitution	Locality
O. officinalis	Zhou-198[a]	24	CC	China
O. eichingeri	IRGC 101144	24	CC	Uganda
O. alta	IRGC 100161	48	CCDD	Brazil
O. australiensis	IRGC 105263	24	EE	Australia

[a] Collected in Guangxi Province, China, in 1994

Table 2 The genomic probes and the stringency used in this study. The stringency was calculated by the equation described by Meinkoth and Wahl(1984). The GC content (50–70%) was estimated by 20 gene sequences of *O. sativa* from GenBank, and the probe length varied between 200 bp and 600 bp, as judged by running a mini-gel

Slide	Probes	Hybridization stringency	Post-hybridization stringency
O. alta	DIG (EE,*O.australiensis*) BIO (CC,*O. officinalis*)	65–75%	50–60%
O. alta	DIG (EE,*O.australiensis*) BIO (CC,*O. officinalis*)	65–75%	78–86%
O. alta	DIG (CC,*O. eichingeri*) BIO (EE,*O. australiensis*)	65–75%	96–100%

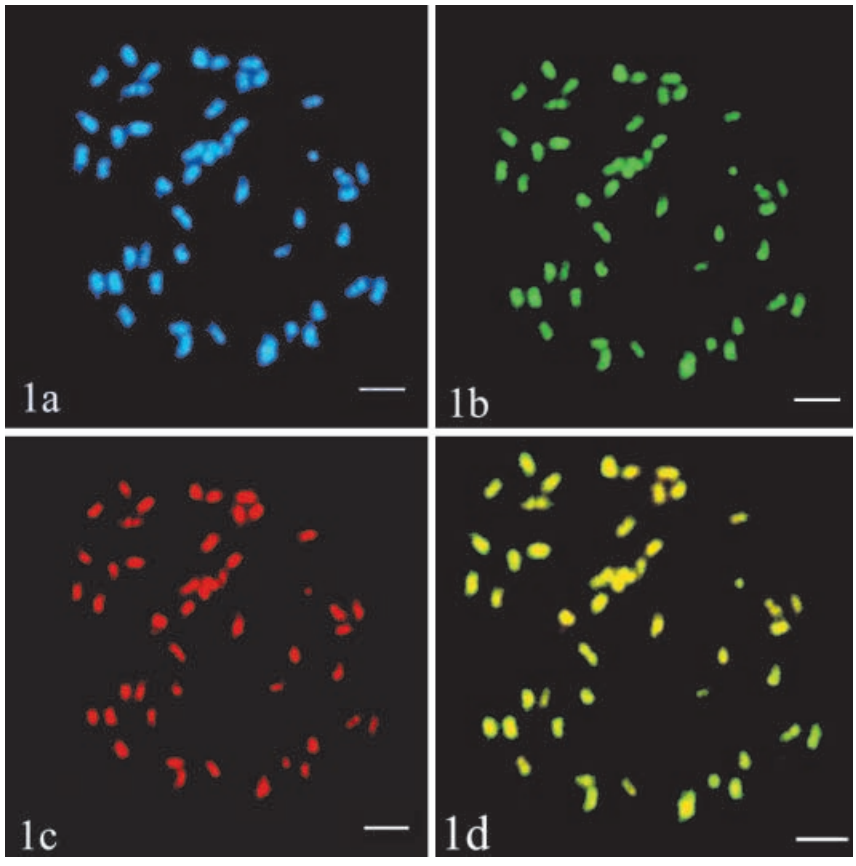

Fig. 1a–d GISH of *O. alta* probed with the E and C genomes. GISH results with 50–60% stringency. **a** Chromosome spreads of *O. alta* stained with DAPI. **b** The chromosomes probed with biotinylated genomic DNA from *O. officinalis* (C genome). **c** The same chromosomes probed with digoxigenin-labeled total DNA from *O. australiensis* (E genome) and detected with rhodamine. **d** A combined image of both **b** and **c**

between 78 and 86%. Figure 2a shows the 48 chromosomes of *O. alta* stained by DAPI. Figure 2b shows the hybridization signals excited by C-genomic probes (from *O. officinalis*). Signals of variable strength could be observed on all chromosomes and, the accordingly, the identity of the chromosomes could be recognized through the fluorescent intensity. The chromosomes painted with stronger fluorescent signals belong to the C genome (arrows), while the rest might belong to the D genome. Nevertheless, some chromosomes could not be easily distinguished as belonging to the C or D genomes, due to weak differences in fluorescent intensity. The result indicates minor differentiation between the C and D genomes of *O. alta*. Figure 2c is a combined image of 2a and 2b. The hybridization signals did not cover the entire length of chromosomes, but only some specific regions, indicating a differentiation between the C genome of *O. alta* and the C genome of *O. officinalis*. Figure 2d shows all chromosomes of the C and D genomes bearing hybridization signals when E-genomic probes were applied, but most hybridization was regional, and only a few chromosomes show strong signals (Fig. 2e). Stronger signals from the E-genomic probe are mainly located on the C-genome chromosomes when compared between Fig. 2b and e. Figure 2f is a McGISH image with probes from both C and E genomes. Generally the C-genome chromosomes show stronger yellowish-green hybridization signals (indicated by arrows) than the D-genome chromosomes.

Figure 3 shows McGISH images, using the C-genomic probes from *O. eichingeri* (showing red signals) and the E-genomic probes (green signals) from *O. australiensis*, with strong post-hybridization washing stringency, between 96 and 100%. At such stringency, almost all non-specific signals would be removed. Figure 3a shows all 48 chromosomes counterstained with DAPI. Figure 3b shows that the chromosomes are painted with red by the C-genomic probes from *O. eichingeri*. The C-genomic probes used in Fig. 3b and Fig. 2b were from different species, but the hybridization pattern is generally similar. By comparing the fluorescence intensity, 20 chromosomes could be clearly identified as C-genome members (white arrows), and the chromosomes indicated by violet arrows are probably the remaining four members of the C genome. The 24 other chromosomes with weak signals should belong to the D genome. Figure 3b also shows that the differentiation between the C and D genomes is minor, even at such high post-hybridization stringency.

When the chromosomes of *O. alta* were hybridized with the genomic probe of *O. australiensis* (EE) at 96 and 100% stringency, the hybridization signals were so weak that further signal amplification was necessary. Figure 3c shows the amplified signals, ranging from strong to very weak, located on all of the chromosomes. Figure 3d is a combined image of Figs. 3a and c. The hybridization sites of the E genomic probe are seen as a blue-green or white color (a mixture of FITC and DAPI

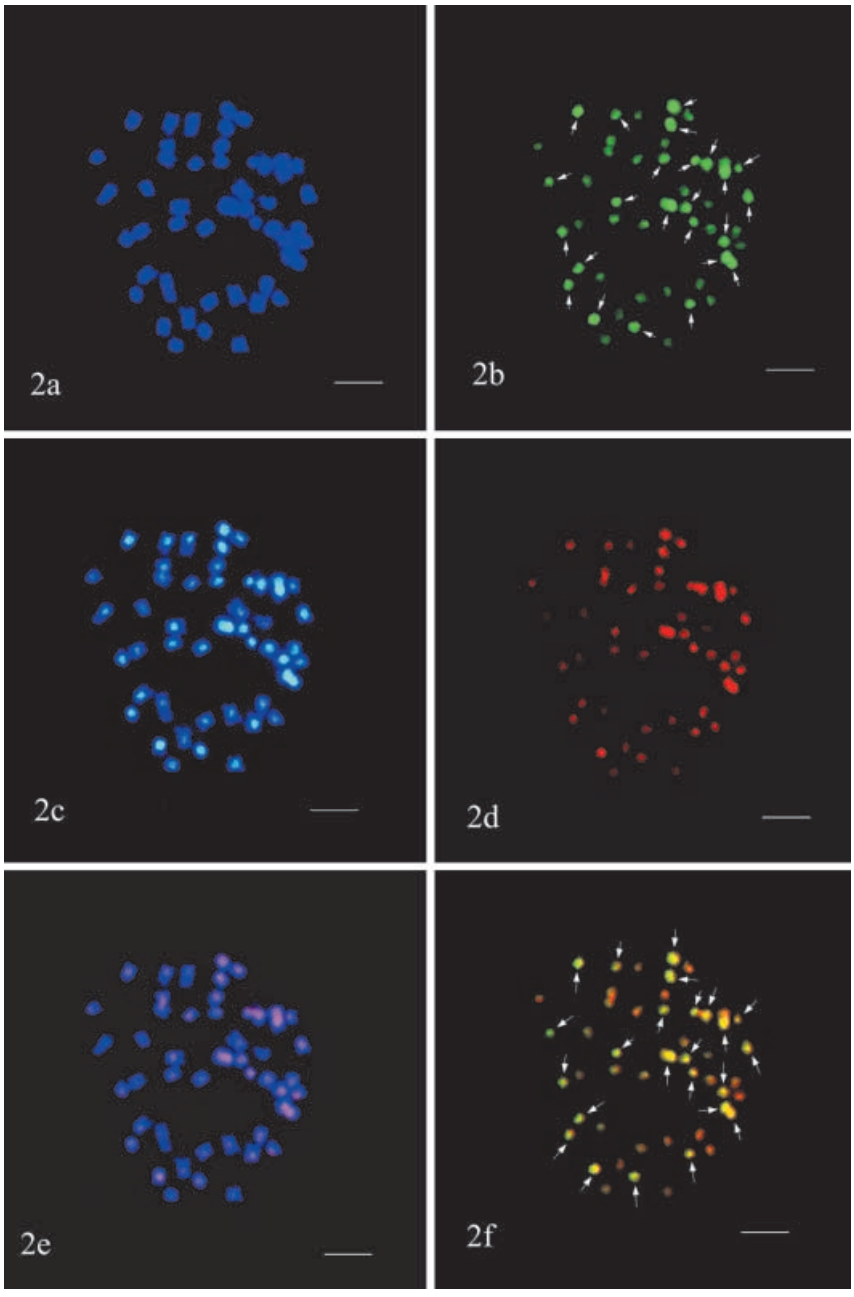

Fig. 2a–f GISH results with 80–85% stringency. **a** Chromosomes stained with DAPI. **b** The same chromosome spread probed with C genomic DNA from *O. officinalis*. The *arrows* indicate the chromosomes belonging to C the genome. **c** A combined image of **a** and **b**, which shows that the hybridization domain only covered a partial region of a chromosome in most cases. **d** The same chromosomes probed with digoxigenin-labelled E-genomic DNA. **e** A combined image of **a** and **d**. **f** The chromosomes hybridized with both E- and C-genomic DNA. The *arrows* indicate the chromosomes belonging to the C genome

fluorescence), and most of them are weak and small, indicating that the E genome shares a relatively small proportion of identical sequences with the C and D genomes. Figure 3e shows the chromosomes hybridized by both the C- and E-genomic probes. The C-genome chromosomes are indicated by arrows, whereas the 20 chromosomes indicated by white arrows obviously belong to the C genome, and the other four indicated by violet arrows most likely belong to the C genome. The hybridization signals derived from the C-genomic probes are red, and those from the the E-genomic probes are yellow (a mixture of FITC and rhodamine fluorescence). The majority of the hybridization signals of E-genomic probe are located on the C- genome chromosomes, indicating that the E genome is more closely related to the C genome than to the D genome. Figure 3f shows a combined image of Figs. 3a and e. The hybridization signals of the C- and E-genomic probes were painted on the background of the chromosomes (counterstained with DAPI). The image shows that it is not possible to distinguish between the C- and D- genome chromosomes precisely. The differentiation between the C genome in *O. alta* (target) and the C genome of *O. eichingeri* (probes) is significant, because the hybridized sites (violet) were localized only on certain regions of the chromosomes, and not along their whole length.

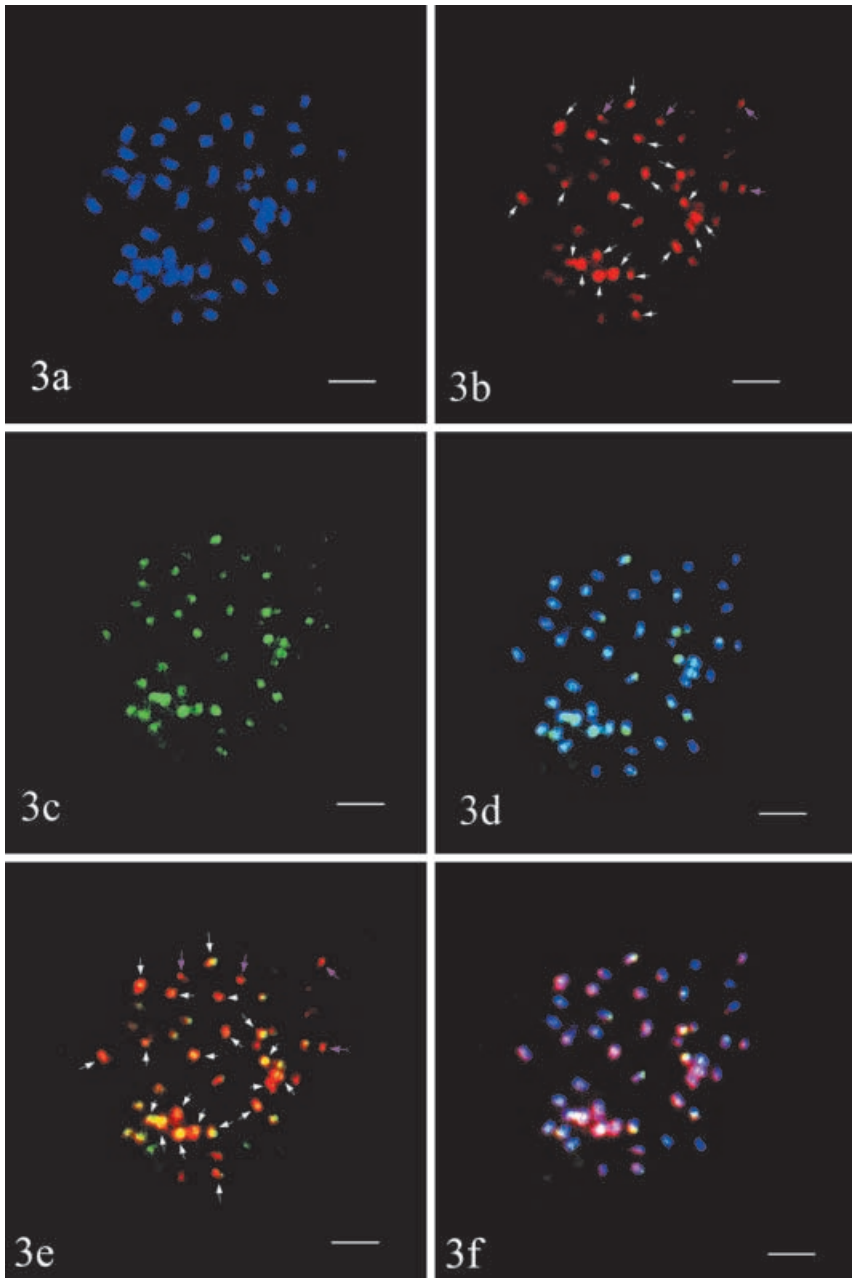

Fig. 3a–f GISH results with 96–100% stringency. **a** The chromosomes stained with DAPI. **b** The same chromosomes probed with C-genomic DNA from *O. eichingeri*. Twenty chromosomes could be clearly identified as the chromosomes of genome C (indicated by white *arrows*), and the chromosomes indicated by violet arrows are probably the other four chromosomes of the C genome. **c** The same chromosomes probed with E genomic DNA. **d** A combined image of **a** and c. **e** A combined image of **b** and **c**. *Arrows* indicate the chromosomes belonging to the C genome. **f** The chromosomes hybridized with both C- and E-genome probes and counterstained with DAPI

Discussion

At lower stringency, the C and D genomes of *O. alta* could not be distinguished from each other when C-genomic DNA from *O. officinalis* was used as a probe. As the stringency was increased (up to 86% and 100%), the signals of C-genomic probes from either *O. officinalis* or *O. eichingeri* were observed on all of the chromosomes of *O. alta*, but the signal intensities were different, varying from stronger to weaker in different chromosomes. The C- and D- genome chromosomes can be distinguished from each other, but it was not possible to identify them accurately (Fig. 2b and Fig. 3b). The results might suggest that the C and D genomes of *O. alta* are slightly differentiated from each other. Fukui et al. (1997) identified D-genomic chromosomes in *O. latifolia* (another tetraploid species with CCDD genomes) by GISH. Probed with labelled genomic DNA from *O. officinalis*, they observed that the difference in the hybridization signal intensity between the C-genome and D-genome chromosomes of *O. latifolia* was small. This indicated relatively high homology in the nucleotide sequences between the C and D genomes. Second (1985a, b) analyzed genome differentiation in *Oryza* through isozyme studies, and found the isozymic patterns of the CCDD species to be different from that of both diploid and allotetraploid

species, but similar to segmental allotetraploids. The results indicated that the CCDD species showed a tendency for minor diploidization. Jena and Kochert (1991) studied the relationship between the C and D genomes through an analysis of C- and D- genomic specific RFLP markers, and concluded that the C and D genomes are closely related. These results are compatible with ours.

Cytogenetic methods were used to detect the relationships among C, D and E genomes, but inconsistent results were obtained. Based on the mean number of paired chromosomes per cell at M1 in the hybrids of *O. australiensis* × *O. alta*, Li et al. (1961,1963) concluded that the majority of bivalents observed in the hybrids might be due to the pairing between the C and D genomes of the CCDD species. Therefore, the C and D genomes of *O. alta* were more-closely related to each other than to *O. australiensis.* This agrees with the results of the present study. However, Katayama (1982) observed that the number of bivalents of the hybrids of *O. officinalis* × *O. australiensis* ranged from 1 to 4; while the hybrids of the amphiploid CCEE × CCDD-genome species ranged from 8 to 24 with a mean of 17.6–20.9 between plants. These results indicated that the E genome was closer to the D genome than to the C genome.

Proximity of the D and E genomes has led to the suggestion that the E genome might have played a critical role in the evolution of the CCDD species (Wang et al. 1992; Ge et al. 1999). However, that suggestion does not agree with the results described in this paper. In our studies, the E-genomic probes can perfectly hybridize with all chromosomes of *O. alta* at 50 to 60% stringency, indicating some sequence similarity of the E-genomic probes and the CD-genomic targets, while at a higher stringency (over 85%) most hybridization sites of the E-genomic probe had small and weak signals on the chromosomes of *O. alta*. Moreover, the strong hybridization signals of the E-genomic probe were mainly located on the C-genome chromosomes, although weak hybridization signals were found on the D-genome chromosomes, indicating that the E genome is closer to C genome than to the D genome. Thus, it seems unlikely that the D genome originated directly from the E genome.

The McGISH results obtained in this paper reveal the impossibility of either the E or C genomes acting as the direct D-genome donor in *O. alta*. The origin of the D genome still remains unknown. The differentiation between the C genome of the diploids (*O. officinalis* or *O. eichingeri*) and the tetraploids (*O. alta*) strongly suggests that neither *O. officinalis* nor *O. eichingeri* was the direct C-genome donor of *O. alta*. Therefore, the origin of the CCDD species still needs further study and justification.

The degree of stringency determines the approximate percent of nucleotides that are correctly matched in the probe and target duplex (Leitch et al. 1994). In the present study, the post-hybridization stringency was designed as a series, ranging from 50 to 100%. The detected signals varied according to the stringency used, and were most-likely related to the degree of differentiation between genomes. This study has shown that adjusting the stringency could provide clear information on the relationships of the related genomes. The results also demonstrate that McGISH hold great potential for identifying differentiated genomes and revealing complicated genomic relationships.

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