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Cytological studies of African cultivated rice, *Oryza glaberrima*

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Abstract African cultivated rice, *Oryza glaberrima* Steud., was cytologically characterized by using both karyotype analysis and molecular cytology. The somatic chromosomes resemble those of Asian cultivated rice, *Oryza sativa* L., in general morphology, although some minor differences were noted. Multicolor fluorescence in situ hybridization (McFISH) with chromosomes detected one 45*s* (17*s*-5.8*s*-25*s*) ribosomal RNA gene locus (45*s* rDNA) and one 5*s* ribosomal RNA gene locus (5*s* rDNA) in the chromosome complement. The 45*s* rDNA and 5*s* rDNA loci were physically mapped to the distal end of the short arm of chromosome 9 and to the proximal region of the short arm of chromosome 11 respectively, as in *O. sativa*. Based on the cytological observations and the physical map of the rDNA loci, the chromosomal organization of *O. glaberrima* and *O. sativa* seems to be very similar.

Key words *Oryza glaberrima* · Karyotype analysis · Ribosomal RNA genes · Image analysis · Multicolor in situ hybridization

Introduction

Oryza glaberrima Steud. is an African cultivated rice mainly distributed in the savanna along the southern fringes of the Sahara desert (Oka 1988). Asian cultivated rice, *O. sativa* L., and *O. glaberrima* have been considered to have the same A genome from cytological observations on the meiotic pairing of their F_1 hybrids (Morinaga 1964). *O. glaberrima*, an endemic species to West Africa which differentiated from the wild rice species *O. barthii* (Clayton 1968) or *O. breviligulata* (Morishima et al. 1963; Oka 1974), has tolerance to various biotic and abiotic stresses (Oka 1988). These characteristics are a valuable potential source of genes for the improvement of *O. sativa*, as well as *O. glaberrima*, although *O. glaberrima* has not yet been improved by modern breeding techniques (Oka 1988). Such an improvement of *O. glaberrima* is now being undertaken by the West African Rice Development Association (WARDA), Bouake, in the Ivory Coast.

Many studies have been carried out on the genetic architecture of *O. glaberrima* (Morishima et al. 1962; Yabuno 1977; Sano 1985). However the cytological features of *O. glaberrima* have not been thoroughly investigated. The only report is on the general similarity between the karyotypes of *O. glaberrima* and *O. sativa* (Hu 1960).

In the present study we describe the detailed chromosomal characteristics of *O. glaberrima* using several new technologies, including image analysis (Fukui 1986, 1988), micromanipulation (Fukui et al. 1992, 1994 a), and molecular cytology (Fukui 1984).

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Materials and methods

Plant material and chromosome-sample preparation

O. glaberrima (W025, kindly supplied by Dr. Y. Sano, National Institute of Genetics, Misima 411, Japan) was grown in a greenhouse. Root tips about 1 cm in length were excised and fixed in ethanol:acetic acid, 1:1, for at least 3 h without any pretreatment.

Chromosome preparations were made by enzymatic maceration of root tips coupled with the air drying method (Fukui and Iijima 1991, 1992; Iijima and Fukui 1991) with the following minor modifications. The root tips were subjected to a decompression treatment for 5–10 min in a modified enzymatic mixture (1% Cellulase Onozuka RS, 0.75% Pectolyase Y-23, 0.15% Macerozyme R200, 0.5 mM EDTA, at pH 4.2) and macerated in a 1.5-ml microtube (Ohmido and Fukui 1995). The chromosomes were stained with a 2% Giemsa solution (pH 6.8) for 15 min.

Chromosomes at resting, prometaphase, and metaphase stages were examined and microphotographed (Axiophot, Zeiss). All the prometaphase chromosomes with a clear condensation pattern (CP, Fukui and Mukai 1988) were identified under the microscope prior to in situ hybridization. The chromosome complements of five good cells at the prometaphase stage were analyzed by imaging methods (Kamisugi and Fukui 1990). Two image parameters, relative length and arm ratio, were measured for all the chromatids and the data were

analyzed statistically. The condensation pattern (CP, Fukui and Mukai 1988) of each chromosome was also checked by the imaging method (Nakayama and Fukui 1994). The chromosomes were then de-stained in 70% ethanol and used for further experiments.

Direct cloning and direct labelling of the 17*s* and 5*s* rDNA of *O. glaberrima*

The 45*s* rDNA consists of a 17*s*-5.8*s*-25*s* rDNA gene cluster in the rice genome. A part of the 17*s* rDNA gene was used as a probe to detect the 45*s* rDNA loci. Digoxigenin-labelled 17*s* rDNA (Dig-17*s* rDNA) and biotin-labelled 5*s* rDNA (Bio-5*s* rDNA) probes were prepared by direct cloning and direct labelling methods. Fixed root tips were subjected to enzymatic maceration/air drying on a ϕ35-mm heat-absorptive, film-lined culture dish. Octagonal disks of root tips, 2 mm in diameter and with about 100 rice nuclei on one disk, were dissected out of the film using an argon-ion microlaser beam through a 100× objective (ACAS470, Meridian). The disk was then transferred into a 0.5-ml microtube and used as a DNA template for PCR (Fukui et al. 1992).

The standard PCR method was carried out using a thermal cycler according to the manufacturer's instructions (Perkin Elmer Cetus). Two pairs of primers, (5′-TAGTCATATGCTTGTCTCAAAGA-3′; 5′-TTGTCACTACCTCCCCGTGT-3′) and (5′-GATCCCATCAGAACTCCGAAG-3′; 5′-CGGTGCTTTAGTGCTGGTATG-3′), were used for the direct cloning and the subsequent direct labelling of the respective 17*s* rDNA (455 bp) and 5*s* rDNA (301 bp) probes (Takaiwa et al. 1984; Fukui et al. 1994 a). The first 30 cycles of the PCR amplified rDNAs directly from the nuclei, while the second 30 cycles simultaneously amplified and labelled the PCR products with either biotin-11-dUTP (Enzo Biochemicals) or digoxigenin-11-dUTP (Boehringer Mannheim Biochemica). The details of the procedure have already been described (Fukui et al. 1994 a; Kamisugi and Fukui, 1995).

Multicolor fluorescence, in situ hybridization (McFISH) and imaging

An improved FISH method developed especially for rice chromosomes (Fukui et al. 1994 b) was employed. The following modifications were applied in the case of simultaneous signal detection of the 45*s* and 5*s* rDNA loci in the chromosomes using different fluorescent colors.

The Dig-17*s* rDNA and Bio-5*s* rDNA were simultaneously hybridized to the chromosomes by applying the hybridization mixture with two probes. The hybridized Dig-17*s* rDNA and Bio-5*s* rDNA were detected by anti-digoxigenin-rhodamine (20 µg/ml, Boehringer) and avidin-FITC (fluorescein isothiocyanate) conjugate (20 µg/ml, Boehringer). FITC signals were enhanced by the secondary immunological reaction of biotinylated anti-avidin (1%, Vector Laboratory, Calif., USA) and fluorescein-avidin (1%, Vector Laboratory, Calif., USA). The chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole, 2 µg/ml, pH 6.8).

Reddish fluorescent signals of rhodamine, yellowish fluorescent signals of FITC, and bluish fluorescent chromosomes of DAPI, were independently observed using a fluorescence microscope with different filter sets (G15, B10, UV01, Axiophot, Zeiss). Three images are separately recorded in the frame memories (768 × 512 pixel matrix with eight bits of gray steps/pixel) of a chromosome image analyzing system II (CHIAS2, VIDAS, Zeiss/Kontron) through a high-sensitive color CCD camera (HCC-3600P, Floubel). After digital enhancement of the signals, the three fluorescence images were integrated into a single image and pseudo-colored to reproduce the actual fluorescent colors by a newly designed look-up-table.

Results

The somatic chromosomes at different stages in the mitotic cell cycle are illustrated in Fig. 1. Figure 1 a shows a nucleus at the resting stage demonstrating chromatin threads and chromomeric granules. The chromomeric granules were scattered throughout the nucleus. The number of heavily stained granules varied between nuclei. Figure 1 b and c show late-prophase and metaphase chromosomes respectively at the same magnification. The late-prophase chromosomes were characterized by uneven condensation due to early and late condensation blocks along the chromosomes, as commonly observed in the chromosomes of the genus *Oryza* and other plants with small chromosomes. The metaphase chromosomes were uniformly condensed to about 2 µm without any pretreatment. A pair of satellite chromosomes was observed as indicated by the arrowheads (Fig. 1 c).

Prometaphase chromosomes are arranged according to a new chromosome numbering system (Khush and Kino-

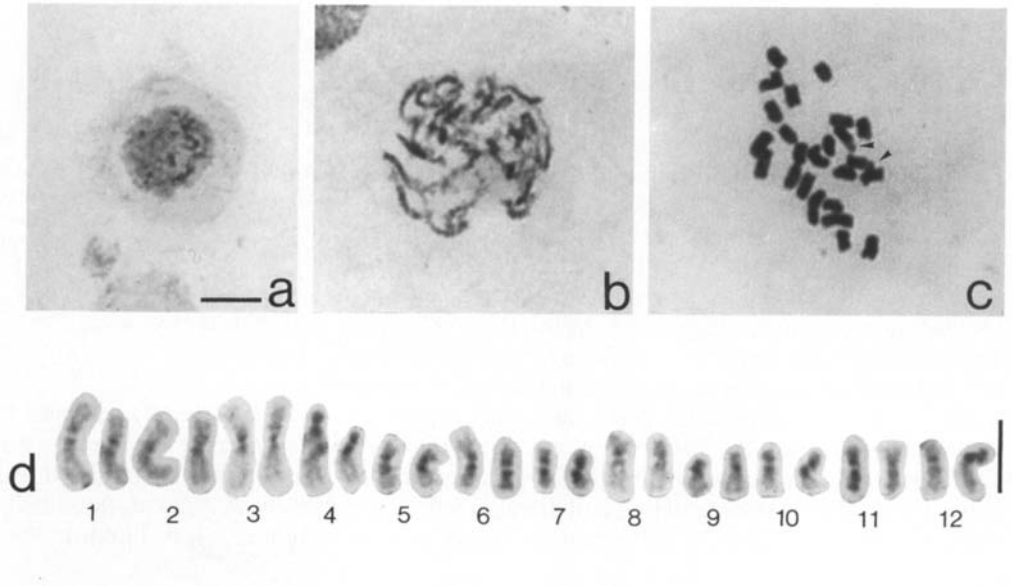

Fig. 1a–d Giemsa-stained somatic chromosomes of *O. glaberrima*. **a** Chromosomes at the resting stage. **b** Chromosomes at the late-prophase stage. **c** Chromosomes at the metaphase stage. **d** Chromosomes at the prometaphase stage. Scale bars represent 5 µm

Table 1 Numerical data of the somatic chromosomes of *O. glaberrima*

Chromosome no.	Relative length (%)[a]	Arm ratio (L/S)[a]	Remarks[b]
1	12.5 ± 0.8** (13.6 ± 1.1)	1.59 ± 0.18 (1.58 ± 0.23)	
2	10.3 ± 0.7 (10.9 ± 0.9)	1.64 ± 0.31 (1.72 ± 0.21)	More metacentric than *O. sativa*
3	11.8 ± 1.3 (11.7 ± 1.2)	1.21 ± 0.21 (1.10 ± 0.25)	
4	9.0 ± 0.5 (9.1 ± 0.6)	3.23 ± 0.24** (2.93 ± 0.29)	
5	6.7 ± 0.5 (6.6 ± 0.4)	1.50 ± 0.13** (1.24 ± 0.13)	
6	8.0 ± 0.7 (8.3 ± 0.6)	1.15 ± 0.12 (1.12 ± 0.09)	Clear FUSC at the distal end of an arm
7	6.6 ± 0.7 (6.1 ± 0.4)	1.19 ± 0.16 (1.26 ± 0.12)	
8	7.8 ± 0.7 (7.6 ± 0.5)	1.43 ± 0.17 (1.61 ± 0.31)	Slit was observed in the distal region of long arm
9	6.4 ± 0.5 (5.8 ± 0.6)	3.61 ± 0.62 (3.90 ± 1.11)	Much larger and more distinct satellite than *O. sativa*
10	6.4 ± 0.8 (5.8 ± 0.4)	1.82 ± 0.44 (2.00 ± 0.37)	
11	7.0 ± 0.3 (6.6 ± 0.5)	1.21 ± 0.19 (1.26 ± 0.11)	Smaller telomeric condensation
12	7.6 ± 0.8 (7.9 ± 0.6)	1.67 ± 0.72 (1.68 ± 0.21)	No FUSC at the terminal of short arm and a FUSC observed on the long arm

[a] Mean ± SD. The length of the satellite was excluded from the calculation. Figures in parentheses indicate the values of *O. sativa* (Fukui and Iijima 1991)
[b] Chromosome characteristics specific to *O. glaberrima*
** Significantly different between *O. glaberrima* and *O. sativa* at the 1% level

shita 1991; Kinoshita 1991) in Fig. 1 d. As in *O. sativa* (Iijima et al. 1991; Fukui and Iijima 1992) each chromosome could be identified based on the condensation pattern of the prometaphase chromosomes.

The general features of prometaphase chromosomes were the same as those of *O. sativa*. Most of the *O. glaberrima* chromosomes were not significantly different from those of *O. sativa* in relative length and arm ratio (Table 1), although the relative length of chromosome 1 and the arm ratios of chromosome 4 and 5 differed significantly at the 1% level. Several other minor differences were noted, and are also listed in Table 1. The FUSC (faint unstable small condensation) (Iijima et al. 1991), which was observed in *O. sativa* chromosomes, was also detected in *O. glaberrima*, especially at the telomeric positions of chromosomes 6 and 12. The satellite of chromosome 9 in *O. glaberrima* was much more distinct than that of *O. sativa*.

Figure 2 shows a microdissection of the template disks and the agarose-gel electrophoresis of the PCR products. Figure 2 a demonstrates the laser dissection of the polyester film to obtain disks with about a hundred nuclei on the surface. The disk holes at the right hand side of the dissected disks show that the disks have already been removed from the film and are then recovered in a 0.5-ml microtube. The results of direct cloning and direct labelling are shown in Fig. 2 b. Lane M is the molecular-size marker

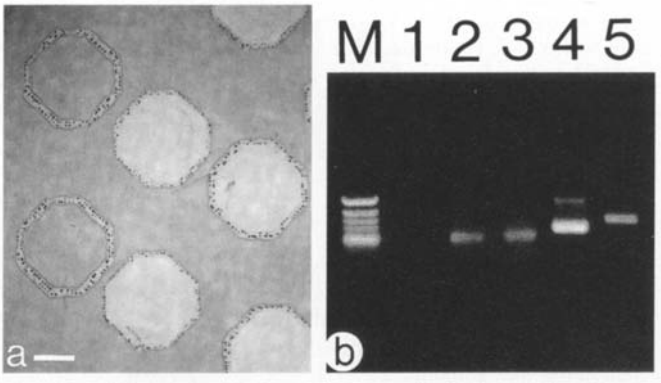

Fig. 2a, b Microdissection and direct cloning of 5*s* and 17*s* rDNAs. **a** Dissection of the octagonal disks with about 100 nuclei. Scale bar represents 1 mm. **b** Agarose-gel (1%) electrophoresis of the products by direct cloning and direct labelling. *Lane M*, molecular maker, ϕX174/*Hin*cII; *lane 1*, negative control; *lane 2*, 5*s* rDNA; *lane 3*, biotin-labelled 5*s* rDNA; *lane 4*, 17*s* rDNA; *lane 5*, digoxigenin-labelled 17*s* rDNA

(ϕX 174/*Hin*cII) and lane 1 is a negative control where a disk without any nucleus was subjected to the PCR. Lanes 2 and 3 show amplified and labelled 5*s* rDNAs, respectively. A retardation of the band in lane 3 indicates the incorporation of biotin to the amplified 5*s* rDNA. Lanes 4

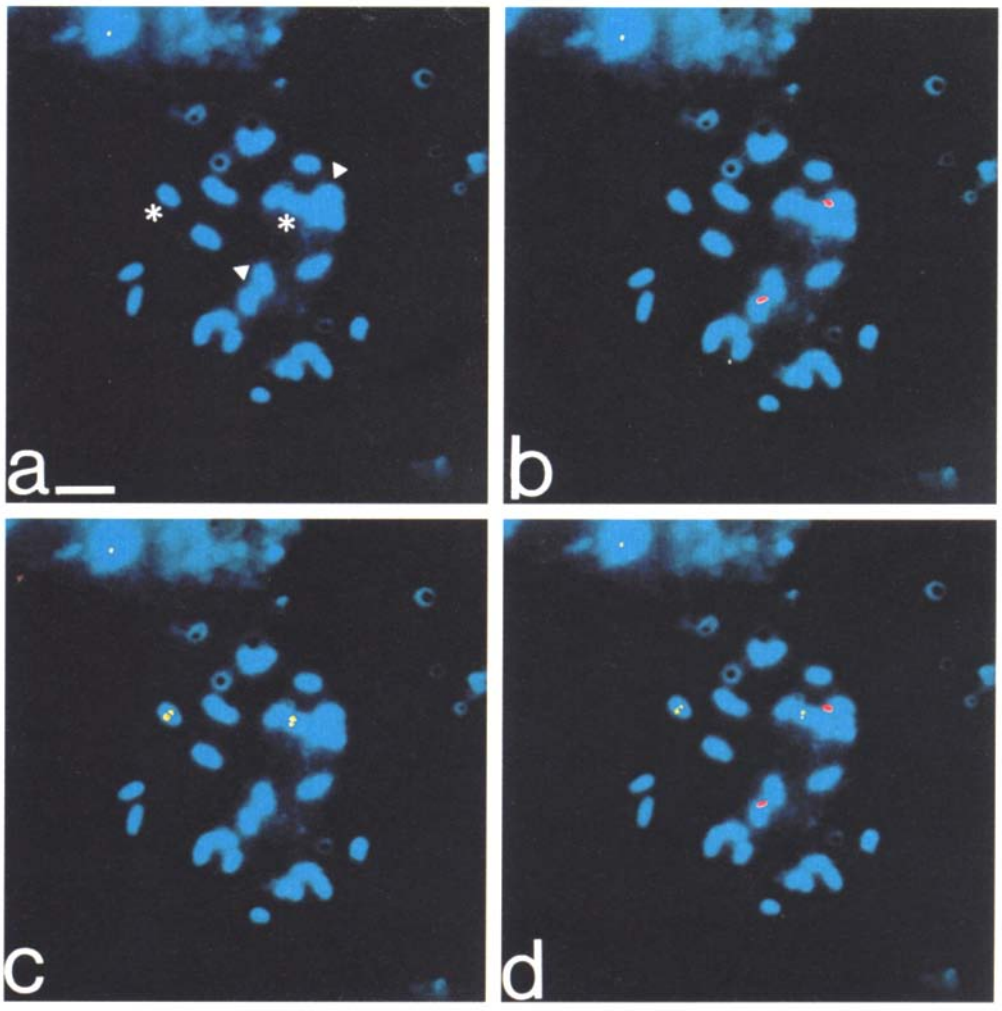

Fig. 3a–d McFISH of *O. glaberrima* chromosomes with simultaneous 5*s* rDNA and 17*s* rDNA probes. **a** DAPI image of interphase chromosomes by UV excitation light. Two pairs of chromosomes, 9 and 11, are indicated by either a *solid arrow head* (▶) or a star (*), respectively. **b** Rhodamine image of the 17*s* rDNA signals in G excitation light. **c** FITC image of 5*s* rDNA signals in B excitation light. **d** Integration of the three images by imaging methods. Scale bar indicates 5 μm

and 5 correspond to the direct cloning and direct labelling of 17*s* rDNA with digoxigenin. The same tendency is observed more clearly in the case of 17*s* rDNA.

McFISH simultaneously revealed the number and position of both the 45*s* and 5*s* rDNA loci using the Dig-17*s* rDNA and Bio-5*s* rDNA respectively as probes (Fig. 3). Figure 3 a illustrates the DAPI image of the *O. glaberrima* chromosomes. All the chromosomes are readily distinguishable since all of them have already been identified prior to McFISH. One pair of *O. glaberrima* chromosomes has three heavily condensed regions and thus corresponds to chromosome 11 of *O. sativa* (Trimodal, Iijima et al. 1991). The chromosomes showing the secondary constriction have been identified as those corresponding to chromosome 9 (Sat-chromosome) of *O. sativa* (Iijima et al. 1991), since the satellites are clearly observed in the Giemsa-stained image of these chromosomes.

Figure 3 b shows an integration of the DAPI-stained chromosome image (Fig. 3 a) with the reddish signal image of a 45*s* rDNA locus. Two sites of the 45*s* rDNA locus are clearly detected at the distal end of the short arm of chromosome 9. In the case of 45*s* rDNA, the doublet signals were not observed, but the single fused signal and/or extended signal were evident at the secondary constrictions, indicating the 45*s* rDNA locus to be the same as the nucleolar organizing region.

Figure 3 c shows an integration of the DAPI-stained chromosome image and the yellowish signal image of the 5*s* rDNA locus. Two 5*s* rDNA sites are clearly observed on chromosome 11. Detailed observation confirmed the locus to be positioned at the proximal region of the short arm of chromosome 11. Doublet formation of the 5*s* rDNA signals is evident in most cases indicating the existence of the 5*s* rDNA locus in each replicated chromatid. Figure 3 d shows the digital integration of the three fluorescent images of the chromosome and the two signal images. One 45*s* rDNA and one 5*s* rDNA locus are simultaneously detected on chromosomes 9 and 11 respectively under the McFISH conditions employed.

Figure 4 depicts the locations of both the 45*s* and 5*s* rDNA loci on the quantitative idiogram of *O. sativa* (Fukui and Iijima 1991) since, as shown in Table 1, most of the relative lengths and arm ratios of *O. glaberrima* chromosomes were not significantly different from those of *O.*

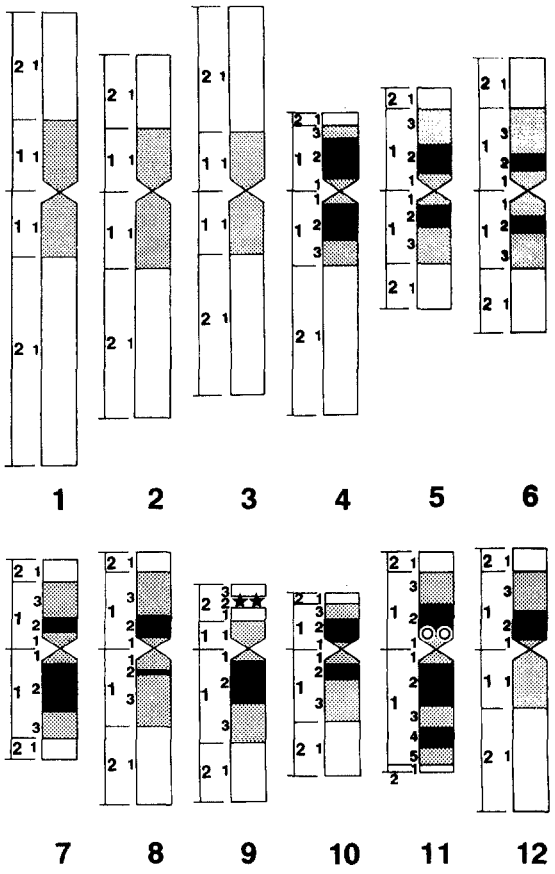

Fig. 4 Chromosome map of the 45*s* rDNA (★) and 5*s* rDNA (○) loci in *O. glaberrima*

sativa. Moreover, the condensation patterns of the *O. glaberrima* prometaphase chromosomes did not differ from those of *O. sativa* by either visual inspection or by the imaging methods. The position of the 45*s* rDNA and 5*s* rDNA loci thus demonstrates that the two ribosomal RNA gene clusters in *O. glaberrima* and *O. sativa* are at similar locations.

Discussion

The detailed cytological characteristics of *O. glaberrima*, as detected through chromosome karyotyping and McFISH, have been presented. Karyotype analysis reveals that the morphology of *O. glaberrima* chromosomes is comparable to that of *O. sativa* (Fukui and Iijima 1991, 1992). The fact that *O. glaberrima* and *O. sativa* ssp. *japonica* have the same number and location for both the 45*s* rDNA and 5*s* rDNA loci in the genome is further evidence of the similar nature of the chromosomal organization of the two species. The number of 45*s* rDNA loci is known to be variable within the A-genome species. *O. sativa* ssp. *indica* and some *O. rufipogon* accessions have two 45*s* rDNA loci (Fukui et al. 1994 b). The basic similarity of the karyotype of both species should be helpful in establishing a complete trisomic series for *O. glaberrima*, which has not yet been developed (Ishiki 1992). The normal behaviour of meiotic chromosomes of the hybrids between *O. sativa* and *O. glaberrima* (Morishima and Kuriyama 1957) and the available genetic studies further support the conclusions of the present paper. Sano (1979) induced several mutations in *O. glaberrima* by EMS treatment and found that these were identical to mutations obtained in *O. sativa*. For example, a glutinous mutant appeared to be allelic to the *wx* mutant of *O. sativa*. Oka (1988) suggested that the two species have a similar genetic architecture on the basis of his genetic analysis of ligulelessness, long empty glume, brittle culm, short culm and the results of comparative mutation research.

Prometaphase chromosome samples without cytoplasmic debris, prepared by the enzymatic maceration/air drying method, made it possible to examine minute differences in chromosome structure and some minor differences were detected between the chromosomes of *O. glaberrima* and *O. sativa*. The telomeric FUSCs, which are also observed in *O. sativa* ssp. *japonica* (Iijima et al. 1991), are one of the differentiating characteristics between these two species. An A-genome-specific repeated sequence (Ohtsubo et al. 1991) hybridized in situ to the telomeric FUSCs and the number of chromosomes involved was three in *O. glaberrima* and two in *O. sativa* ssp. *japonica* (unpublished data). The size of the satellite in *O. glaberrima* was also larger than that of *O. sativa*. These results suggest that the chromosomal organization of *O. glaberrima* and *O. sativa* has been subjected to some modification, especially at the distal regions of the chromosomes, during speciation.

McFISH has been successfully employed in rice using directly cloned and labelled DNA probes. This opens the way, not only for more effective mapping of different DNA sequences in the rice chromosomes, but also for determining the order of different clones within a chromosome (cf. Montogomery et al. 1993). It is anticipated that a number of different DNA sequences will be detected by further improvements in McFISH. Estimation of the copy number of the genes at a locus and localization of single-copy genes are also likely to be achieved in the near future.

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