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Variability in rDNA loci in the genus *Oryza* detected through fluorescence in situ hybridization

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Abstract. The 17*s*–5.8*s*–25*s* ribosomal RNA gene (rDNA) loci in *Oryza* spp. were identified by the fluorescence in-situ hybridization (FISH) method. The rDNA loci were located on one-to-three chromosomes (two-to-six sites) within the eight diploid *Oryza* spp. One of the rDNA loci gave the weakest hybridization signal. This locus is reported for the first time in the genus *Oryza*. The chromosomes containing the rDNA loci were determined to be numbers 9, 10 and 11 in descending order of the copy number of rDNA. The application of image analysis methods, after slide preparation treatments (post-treatments), and the use of a thermal cycler, greatly improved the reproducibility of the results. The evolutionary significance of the variability of rDNA loci among the *Oryza* spp. is discussed.

Key words: Fluorescence in-situ hybridization (FISH) – Ribosomal DNA – Genus *Oryza* – Image analysis – NOR variability

Introduction

Rice chromosomes with a large 17s–5.8s–25s ribosomal RNA gene (rDNA) array have been identified as the satellite chromosomes by their characteristics (Fukui and Iijima 1991; Yanagisawa et al. 1991). They are also recognized as the chromosomes with nucleolar organizing regions (NOR chromosomes).

In cultivated rice, *Oryza sativa* L. ssp *japonica*, one pair of NOR chromosomes was reported by Kurata and Omura (1978) and Fukui and Iijima (1991). This NOR chromosome was designated as no. 11 (Fukui and Iijima 1991) but according to the new system for numbering rice chromosomes, has now been redesignated as no. 9 (Khush and Kinoshita 1991; Fukui and Iijima 1992). By contrast, two pairs of NOR chromosomes were reported in *O. sativa* ssp. *indica* (Wu et al. 1985).

Although the rDNA-containing chromosomes show conspicuous characteristics as satellite chromosomes, they are sometimes difficult to identify morphologically when the copy number of the rDNA units at the locus is small (Leitch and Heslop-Harrison 1992). The in-situ hybridization (ISH) method (Appels et al. 1980; Hutchinson and Miller 1982; Rayburn and Gill 1985) offers a way out of this impasse since it is based on the detection of rDNA loci directly by molecular hybridization. Using this technique one rDNA locus was identified on chromosome 9 in japonica rice. (Fukui et al. 1987; Fukui 1990; Iijima et al. 1991) while two rDNA loci were detected in indica rice (Islam-Faridi et al. 1990).

Although ISH is now widely employed in cytogenetic analysis, it is time consuming and strict experimental protocols are needed for its success. Therefore we have developed a reproducible and convenient fluorescence ISH (FISH) technique in conjunction with imaging methods, the use of a thermal cycler, and various post-treatments. As a result, clear fluorescent signals were reproducibly obtained and a new rDNA locus was detected in two diploid wild rice species.

Materials and methods

Plant materials and cytological procedures

Nine rice species, as listed in Table 1, were obtained either from the gene bank of the National Institute of Genetics (Mishima

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411, Japan) or Hokuriku National Agricultural Experiment Station (Joetsu 943-01, Japan). Seeds of trisomic lines for chromosomes 9 and 10, and their original variety, IR24, were supplied by Dr. Tsugufumi Ogawa (Kyushu National Agricultural Experiment Station, Chikugo 830, Japan). Seeds were germinated on moist filter paper in Petri dishes at 27 °C under continuous illumination. Root tips 1–2 cm long were excised and fixed in ethanol : acetic acid (1 : 1). They were stored at −20 °C for about 1 week before examination.

The procedures for sample preparation were according to the protocol described by Fukui and Iijima (1991, 1992) with minor modifications as follows: (1) the glass slides which were used for in-situ hybridization were coated with a 0.1% poly-L-lysine solution (Sigma); (2) enzymatic maceration was carried out in a 1.5-ml Eppendorf tube at 37 °C for 60–90 min; (3) the root tips were treated for 15–30 min in a decompression chamber before maceration and (4) the chromosome preparations were flame-dried.

Fluorescence in-situ hybridization (FISH)

Chromosome samples on a glass slide were subjected to four sequential 'post-treatments'. Firstly, they were treated with an enzymatic mixture (2% Cellulase Onozuka RS, Yakult Honsha, Co., Ltd., Tokyo, 1.5% Macerozyme R-200, Yakult Honsha, and 0.3% Pectolyase Y-23, Seishin Pharmaceutical Ltd., Tokyo, 1mM EDTA, pH 4.2) in 2 × SSC at 37 °C for 30 min. Secondly, they were treated with 1 mg/ml Proteinase K (Wako Pure Chemical Industries Ltd., Osaka) at 37 °C for 30 min. Thirdly, they were washed in 45% acetic acid for 5 min. They were completely dehydrated through a 70, 95 and 99% ethanol series for 10 min each and were air-dried. Finally, they were treated with 100 µg/ml RNase A (Sigma) in 2 × SSC at 37 °C for 60 min.

The rDNA probe (Sano and Sano 1990) was kindly supplied by Dr. Yoshio Sano, National Institute of Genetics, Mishima 411, Japan. This probe is 3.8 kb in length and covers most of the coding regions of the ribosomal RNA genes and the flanking spacer regions. The probe was labelled by a random primer labelling method with biothin-dUTP under the supplier's instructions. A 15-µl aliquot of the hybridization mixture containing 100 ng of biotinylated-rDNA in 50% formamide/2 × SSC was dropped on a glass slide. The solution was covered with a cover slip, sealed with liquid Arabian gum and then air-dried.

The glass slide with sealed cover slip was placed on a thermal cycler (PHC-3, Techne, Cambridge, UK) that had been remodelled by adding an 80 × 120 mm cast-aluminum flat plate. The programmed heating sequence was 70 °C for 6 min and 37 °C for 18 h.

The cover slips were removed and the slides were washed with 2 × SSC three times and once with 4 × SSC at 37 °C for 10 min each. A 70-µl aliquot of fluorescein isothiocyanate (FITC)-avidin conjugate (0.1 mg/ml, Boehringer Mannheim) was dropped onto the glass slides, which were then incubated at 37 °C for 60 min. After rinsing the FITC-avidin solution with BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) three times at 40 °C for 10 min each, a 70-µl biotinylated anti-avidin solution (1%, Vector Laboratory, Calif., USA.) was dropped onto the glass slides which were then incubated at 37 °C for 30 min. After brief washing with BT buffer, a 70-µl fluorescein-avidin solution (1%, Vector Lab.) was applied to each slide. The slides were again incubated at 37 °C for 30 min and then washed thoroughly with BT buffer three times at 40 °C for 10 min each.

Blocking was carried out three times before probe hybridization and before the immunological reaction with 5% bovine serum or goat serum albumin in BT buffer at 37 °C for 5 min.

The slides were counter-stained with a propidium iodide (PI) solution (50 µg/ml in phosphate buffer, pH 6.8, 12.5 mg/ml *p*-phenylendiamine dihydrochloride, with 90% glycerol) and were then examined by fluorescence microscopy.

Fluorescence microscopy and image analysis

A fluorescence microscope (Axiophot, Zeiss) with B- and G-light excitation filters (B10, G15) was used. A highly-sensitive color CCD camera (HCC-3600P, Floubel, Tokyo) was mounted on the microscope and the fluorescent images were directly frozen in the image frame memories of an image analysis system (VIDAS, Zeiss). All the B- and G-light excitation images were separately recorded in floppy disks and were subjected to image analysis.

Each image has 512 × 512 pixel matrix with 256 steps of a grey value for each pixel as in the images previously analyzed and reported by using the chromosome image analyzing system, CHIAS (Fukui 1985, 1986, 1988; Fukui et al. 1989). Necessary image manipulations consisting of shading correction, normalization, binalization, application of the median filter, and erasing of noise on the binary image, were carried out.

Details of each image filter and image manipulation were as reported previously (Fukui and Kakeda 1990; Fukui and Iijima 1991). Chromosomal areas and the signal regions were separately extracted from the respective G- and B-light images. For the fluorescent signals obtained in the B light, the original grey values were transformed to grey values ranging from 200 to 255. The grey values ranging from 0 to 199 were allocated to the pixels of the G-light image that demonstrated mainly chromosome images. The two grey images were combined into a single image.

Pseudocoloration using a look-up-table increased the definition of the image due to the differential coloration generated by computer imaging. The look-up-table was developed by trial and error by repeatedly comparing the original microscopic images with the computer-generated images (Fukui and Ito 1989; Fukui and Kamisugi 1991). The original source images both in B and G light were photographed using reversal color films (Fujichrome 100, ISO 100, Fuji Photo Film Co., Ltd., Tokyo). Digital images were photographed by a color image recorder (CIR-310 Nippon Avionics, Ltd., Tokyo) using reversal color films (Ektachrome 100, ISO 100, Kodak).

Results

Figure 1a shows the G-light excitation image of the chromosomes of *O. sativa*, ssp, *indica*, cv IR36. Two pairs of fluorescent signals were observed in B light (Fig. 1b). Figure 1c shows the intergrated image obtained by image manipulation. The current B or G excitation filter used in the experiment visualized either the yellowish-green fluorescence of FITC/fluorescein or the reddish fluorescence of PI. By image processing, the two fluorescent signals were integrated into a single image with yellowish signals on the reddish chromosomes. For basic information on the size and number of signals on the chromosomes, the visual recognition of the integrated image was markedly improved by image processing as shown in Fig. 1c. The four signal positions of IR36 were more precisely determined by using the integrated image compared with the two original images.

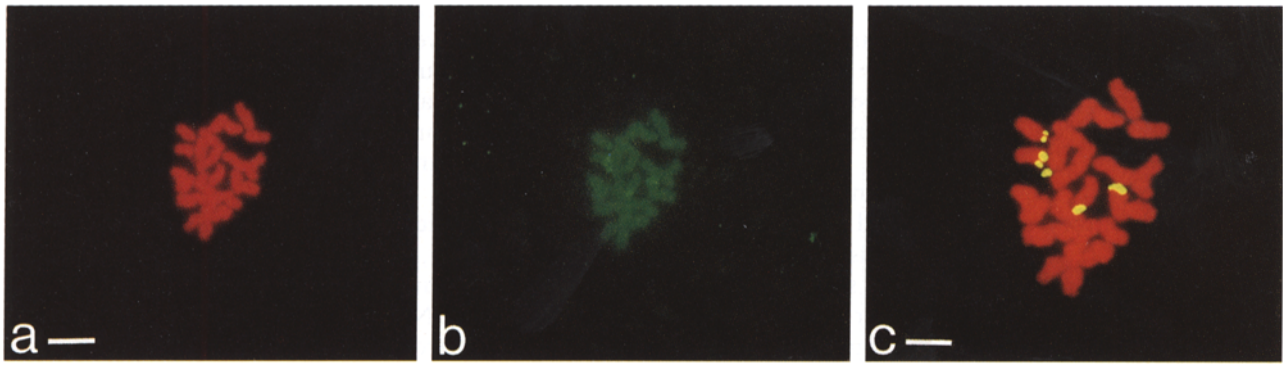

Fig. 1a–c. In-situ hybridization of IR36 chromosomes using the biotinylated rDNA probes. **a** Original photographic image of the chromosomes in G light. **b** Original photographic image of signals in B light. **c** Integrated image of both a and b images by imaging methods. The image was also digitally zoomed up. Bars indicate 5 μm for **a** and **b** and 3 μm for **c**

Figure 2 depicts eight representative examples of signal occurrence on the chromosomes in the nine *Oryza* species with six different genomes. The rDNA sites varied from one pair (Fig. 2a, g), two pairs (Fig. 2b, e, f), three pairs (Fig. 2c, d) and five pairs (Fig. 2h) within the chromosome complements. Although the size and intensity of the signals varied from sample to sample, a general pattern of signal size and intensity was evident. Two large and two medium-sized signals were most commonly observed in the

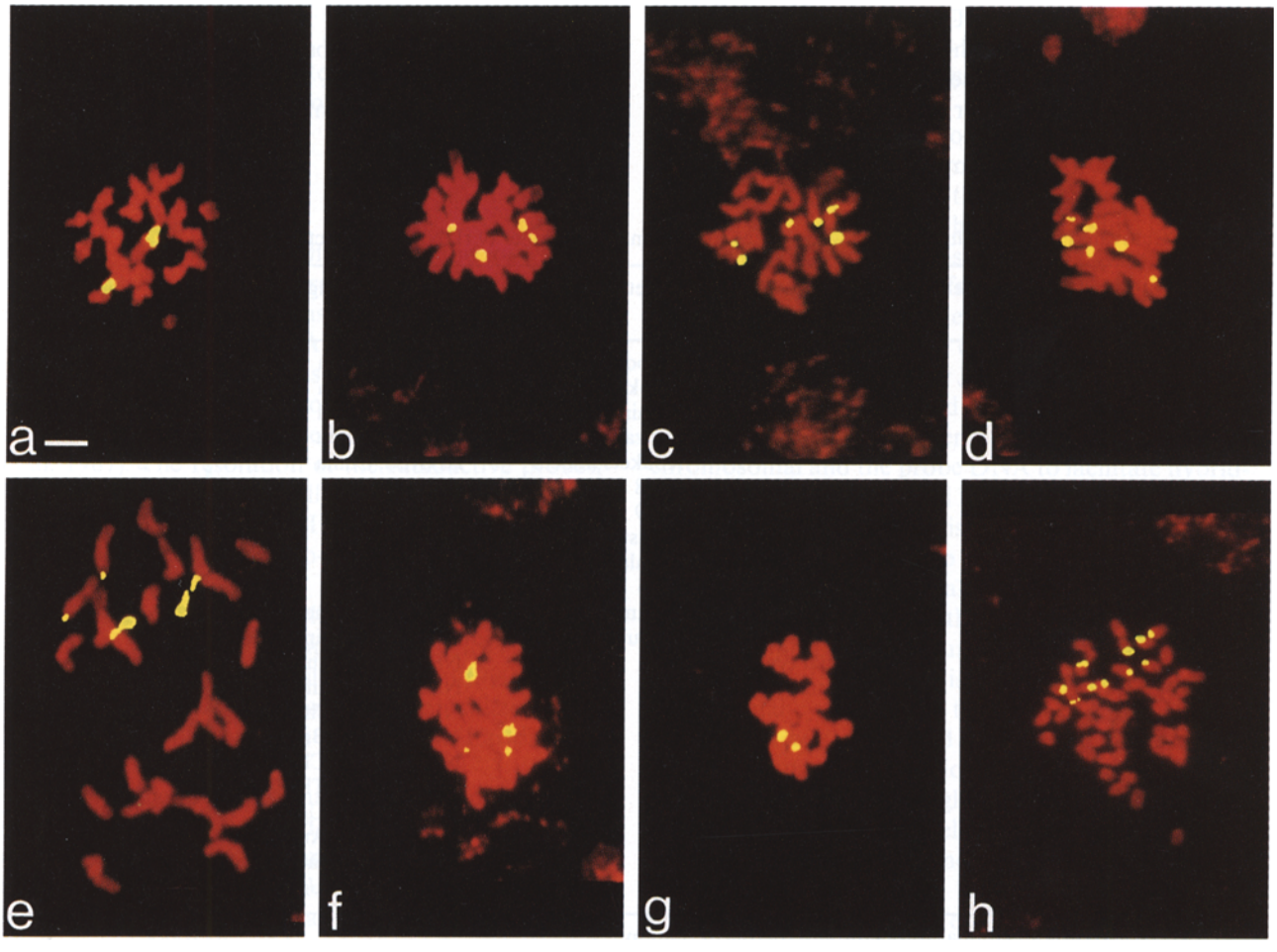

Fig. 2a–h. Fluorescent signals of rDNA sites in eight rice species. **a**, *O. sativa* ssp. *japonica*, CH79; **b**, *O. sativa* ssp. *javanica*, Inakupa; **c**, *O. punctata*; **d**, *O. officinalis*; **e**, *O. eichingeri*; **f**, *O. australiensis*; **g**, *O. brachyantha*; **h**, *O. latifolia*. Bar indicates 3 μm

species, giving the four signals as demonstrated in Fig. 2e and f. Two large-, two medium-, and two small-sized signals were observed in the species with six signals, although the differences between the large- and medium-sized signals were not always clear (Fig. 2c, d).

On occasions the secondary constriction, the adjoining part of the chromosome and the satellite were all fluorescent (Fig. 2e). Such signals were sometimes counted as two for one rDNA site, so that the signal number was sometimes three, four or even six (Fig. 2e). It was, however, possible to distinguish two genuine signals from the situation where two signals were created at one site by the chromosomal morphology observed in G excitation light. The secondary constrictions were also sometimes fluorescent (Fig. 2e).

Table 1 summarizes the number of rDNA loci detected by the FISH method in nine *Oryza* species with the A, A_{gp}, B, C, E, F and CD genomes. One or two rDNA loci were identified in the species with the A genome. One B genome species showed three rDNA loci. Two C genome species showed either two or three rDNA loci. Species with the E and F genomes exhibited either two or one rDNA loci, respectively. The number of rDNA loci in the D genome may be either two or three as in the case of the C genome species. Variability in the number of rDNA sites was thus observed between the species with different genomes, as well as between species with the same genome. Moreover the number of rDNA loci differed between the lines within a species.

Table 1 reports the results of a detailed examination of the number of rDNA loci amongst the A genome species.

Three japonica varieties, Nipponbare, Aikoku and Tushimaakamai, had one rDNA locus. Three indica varieties, Chinsurah Boro II, Kasalath and IR36, exhibited two rDNA loci. Two javanica rice varieties, Ketan Nanga from Indonesia and Inakupa from the Philippines, showed two rDNA loci. Three japonica varieties from China showed one rDNA locus. On the the other hand Kouketsumochi, a glutinous rice from southern China, showed two rDNA loci.

O. rufipogon, an A genome wild species, consists of two groups, a perennial type and an annual type (Oka 1983). One annual and two perennial types were examined. One or two rDNA loci were observed in the perennial. Two rDNA loci were detected in the annual.

Figure 3 shows the identification of the rDNA chromosomes by the FISH method using two trisomic lines and the parental variety, IR24. Two large- and two small-sized signals were observed on the chromosomes of disomic IR24 (Fig. 3a). Five signals were however discernible in both trisomic lines. All the fluorescent

Table 1. Cultivated rice (*O. sativa*) and the wild species studied and the number of rDNA loci detected

Species	Genome	Varietal group	Variety name	Source[a] and origin	Number of rDNA loci
O. sativa	AA	*japonica*	Nipponbare	HNAES, Japan	1
			Aikoku	HNAES, Japan	1
			Tushimaakamai	HNAES, Japan	1
			Tarizaohsien	HNAES, China	1
			Kouketsumochi	HNAES, China	2
			Ch78	NIG, China	1
			Ch79	NIG, China	1
		indica	Chinsurah Boro II	HNAES, India	2
			Kasalath	HNAES, India	2
			IR36	HNAES, India	2
		javanica	Ketan Nanga	HNAES, Indonesia	2
			Inakupa	NIG(221), Philippines	2
O. rufipogon	AA	Annual type		NIG(W0106), India	2
		Perennial type		NIG(W0149), India	2
				NIG(W1944),China	1
O. glumaepatula	A_{gp} A_{gp}			NIG(W1192), Brazil	1
O. punctata	BB			NIG(W1582), Chad	3
O. officinalis	CC			NIG(W0002), Thailand	3
O. eichingeri	CC			NIG(W1521), Uganda	2
O. australiensis	EE			NIG(W1538), Australia	2
O. brachyantha	FF			NIG(W1401), Sierra Leone	1
O. latifolia	CCDD			NIG(W0019), Unknown	5

[a] HNAES = Hokuriku National Agricultural Experiment Station
NIG = National Institute of Genetics
Figures in parentheses indicate the accession number

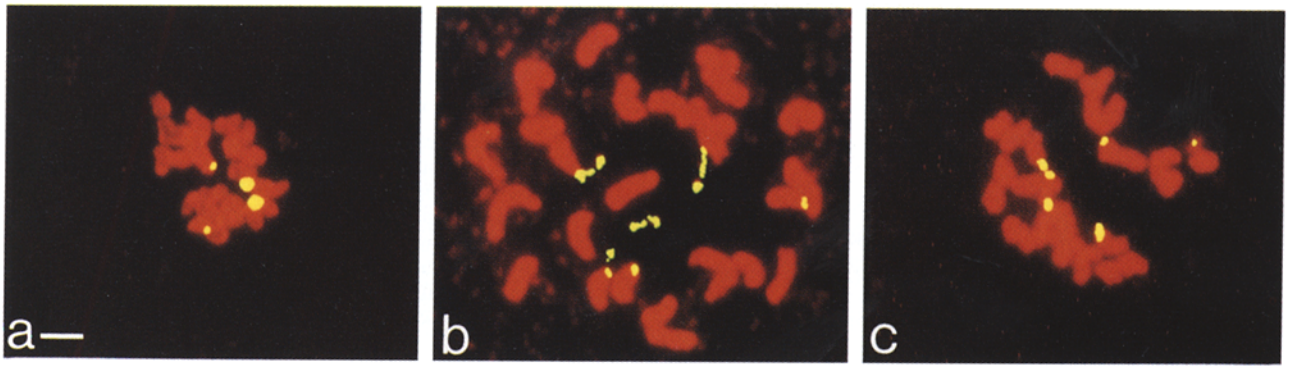

Fig. 3a–c. In-situ hybridization of IR24 and its two trisomic lines. **a**, IR24; **b**, trisomic line for chromosome 9; **c**, trisomic line for chromosome 10. Bar indicates 3 μm

signals were located at the ends of the signal-tagged chromosomes. The trisomic for chromosome 9 displayed five signals: three large and two medium (Fig. 3b), whereas the trisomic for chromosome 10 showed two large- and three medium-sized signals (Fig. 3c). The signal on chromosome 9 was always larger and the intensity stronger than that on chromosome 10. For the third locus, the chromosome involved could not be definitely determined as there was no trisomic line for this chromosome.

Discussion

In situ hybridization is an effective method for localizing specific RNA or DNA sequences on the chromosomes. It has been applied using radioisotope-labelled ribosomal DNA or RNA (Appels et al. 1980; Gerlach and Peacock 1980; Hutchinson and Miller 1982). Successful mapping or rDNA on rice chromosomes was first achieved using ^{125}I-labelled rDNA probes (Fukui et al. 1987). The resolution of the radioactive probes, however, is limited, especially in small plant chromosomes such as those of rice. It also takes a long time to detect the signals by micro-autoradiography (Fukui 1984).

A non-radioactive labelling method with biotin was developed and widely applied to the ISH method (Rayburn and Gill 1985; Heslop-Harrison 1990; Mukai et al. 1990). The rDNA loci of rice were localized using biotinylated probes (Fukui 1990; Islam-Faridi et al. 1990; Iijima et al. 1991).

Mainly due to the difficulty in preparing chromosomes suitable for ISH and the identification of rice chromosomes after ISH, it has been difficult to localize the genes by ISH in rice, although some positive results have been reported (Wu et al. 1986, 1991; Fukui et al. 1987; Fukui 1990; Suzuki et al. 1991; Gustafson and Dillé 1992).

We have made three improvements in the fluorescence ISH (FISH) procedure to obtain reproducibly clear signals on rice chromosomes. Firstly, a combination of post-treatments was introduced to avoid the thin fluorescent layer often observed to cover all the field after FISH treatment, especially under B excitation light. The four post-treatments applied after sample preparation were as follows: (1) digestion of the polysaccharide layer, originating mainly from the debris of the cell walls, by the use of an enzymatic cocktail; (2) removal of chromosomal proteins by proteinase treatment; (3) elimination of the scattered cytoplasmic debris around the chromosomes by washing with 45% acetic acid, which resulted in a considerable reduction of the noise caused by non-specific signals associated with the fragments of cellular debris; and (4) removal of ribonucleoproteins, that are loosely associated with the surface of the metaphase chromosomes (Sato et al. 1988), by RNase treatment.

Secondly, a rearranged thermal cycler was employed throughout the denaturation process of the chromosomal and the probe DNA to facilitate hybridization of the chromosomal DNA with the probes. The most critical step of FISH is the denaturation process of DNA. Usually this step lasts only a few minutes depending on the material. The maintenance of a constant temperature is rather difficult after the glass slides are dipped in a denaturing solution since the heat capacity of glutaraldehyde is limited (data not shown). The thermal cycler affords the most precise heat control since it was developed for the polymerase chain reaction. Thus the fluctuations caused by manual dipping and raising of the glass slide in and out the solutions were practically eliminated.

Thirdly, an imaging method was introduced to analyze the FISH signal. Faint fluorescent signals, observed especially in B excitation light, could be enhanced and integrated into a chromosomal image in G light by the imaging method. The utility of imaging

methods in plant chromosome research was first revealed in 1985 when a chromosome image analyzing system, CHIAS, was developed (Fukui 1985, 1986, 1988; Fukui et al. 1989). Image analysis for chromosome research has been effective in the identification and characterization of rice chromosomes (Fukui and Iijima 1991, 1992; Iijima et al. 1991).

Evolutionary significance of the variability in the number of rDNA loci

This study has revealed variability in the number of rDNA loci among the eight diploid and the one tetraploid species within the genus *Oryza*. Such variability is rare in the *Hordeum* and *Triticum* (Dvorak et al. 1989; Mukai et al. 1991), where rDNA sites have been investigated using the ISH technique.

It is worth noting that wild rice species have a full range of variability as regards the number of rDNA loci. However, *O. rufipogon*, a putative ancestor of cultivated rice, has rDNA variability which is similar to that of cultivated rice. Cultivated rice has either one or two rDNA loci: Varieties in temperate regions have one rDNA locus while those in tropical and subtropical regions have two rDNA loci. It appears that there has been selection pressure to reduce the number of rDNA loci under adverse conditions such as the low temperature prevalent in temperate areas.

Javanica is sometimes referred to as a tropical *japonica* since there is evidence to prove its similarity with *japonica* based on RFLP analysis (Wang and Tanksley 1989; Kawase et al. 1991) as well as morphological similarities (Oka 1958; Sato 1987). Two *javanica* varieties showed two pairs of rDNA loci indicating the similarity to *indica*. These results may be explained by the environmental similarity of the areas where both javanica and indica varieties are grown.

The NOR chromosome in species with one NOR had already been determined as no. 9, both morphologically (Kurata and Omura 1978; Fukui and Iijima 1991, 1992) and by ISH (Fukui et al. 1987; Fukui 1990; Iijima et al. 1991). Another NOR chromosome was identified as no. 10 (Islam-Faridi et al. 1990). Using the trisomic lines for chromosome 9 or 10, the signal intensity for each locus was found to be different. The locus on chromosome 9 is stronger than that on chromosome 10. The third rDNA locus was the weakest among the three rDNA loci. Since there are no trisomic series in species having this locus, it is difficult to identify the chromosome bearing this rDNA locus. It is, however, suspected to be chromosome 11, due to the conspicuous secondary constriction in the interstitial region of its long arm (Fukui and Iijima 1991). The occurrence of a fluorescent signal at this locus in the interstitial part of the long arm of a rather small chromosome in the two wild rice species lends support to this hypothesis.

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