



Title	RICE CHROMOSOMES STUDIES BY FLUORESCENCE IN SITU HYBRIDIZATION WITH SPECIAL REFERENCE TO PHYSICAL MAPPING AND CHROMOSOME STRUCTURE
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Citation	Journal of the Faculty of Agriculture, Hokkaido University, 66(3), 277-320
Issue Date	1995-09
Doc URL	<a href="http://hdl.handle.net/2115/13140">http://hdl.handle.net/2115/13140</a>
Type	bulletin (article)
File Information	66(3)_p277-320.pdf



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# RICE CHROMOSOMES STUDIES BY FLUORESCENCE IN SITU HYBRIDIZATION WITH SPECIAL REFERENCE TO PHYSICAL MAPPING AND CHROMOSOME STRUCTURE\*

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## Contents

I. INTRODUCTION .....	278
II. DEVELOPMENT OF FISH METHOD AND MAPPING OF 45S rDNA LOCI	
A. Introduction .....	280
B. Materials and methods .....	280
C. Results .....	283
D. Discussion .....	285
III. DETECTION OF TANDEM REPEAT TEQUENCES A (TrsA) BY IMPROVED FISH METHOD	
A. Introduction .....	287
B. Materials and methods .....	287
C. Results .....	291
D. Discusson .....	296
IV. CHARACTERIZATION OF <i>ORYZA GLABERRIMA</i> CHROMOSOMES BY McFISH METHOD	
A. Introduction .....	298
B. Materials and methods .....	298
C. Results .....	300
D. Discussion .....	305
V. CONCLUSION .....	306
VI. ABSTRACT .....	308
ACKNOWLEDGEMENTS .....	309
REFERENCES .....	309

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\*Doctoral thesis submitted to the Graduate School of Agriculture, Hokkaido University (1994).

## I. INTRODUCTION

Cytogenetical studies have played an important role to obtain basic information indispensable to plant genetics and breeding. Genome analyses were introduced in the 1930s and 40s, using the pattern of chromosome pairings at meiosis<sup>47)</sup>. Karyotype analysis based on chromosome length and arm ratio has also described plant chromosomes for over 30 years<sup>92)</sup>.

Recently molecular approaches have made remarkable progress in helping study of plant chromosomes. Modern chromosome manipulations, such as construction of chromosome-specific DNA libraries, isolation of specific chromosomes using flow-cytometry<sup>4)</sup>, microdissection of plant chromosomes and direct cloning of ribosomal RNA genes<sup>25)</sup>, have been developed. Recently plant genome research which is aimed at analyzing the whole rice genome has been initiated in Japan after the successful progress of the human genome project.

In the genome research projects of cereal crops, rice (*Oryza sativa*) is a greatly attractive material both in basic and applied science<sup>10)</sup>. Above all, rice is one of the world most important crops in terms of economic and principal food. The genome size of rice is relatively small,  $4 \times 10^8$  bp, among Gramineae<sup>46)</sup>. Twelve linkage groups corresponding to haploid chromosome numbers have been established and the chromosomal basis of each linkage group was facilitated by the production of trisomic series<sup>49)</sup>. Recently molecular maps of rice using restriction fragment length polymorphism (RFLP) have been developed by the efforts of three groups<sup>58,60,83)</sup>. Furthermore, the number of markers mapped on the chromosomes is increasing rapidly since the initiation of the rice genome project in Japan. To understand the structure and function of the rice genes, it is essential to localize the genes or specific DNA sequences, like RFLP and RAPD (random amplified polymorphism DNA) markers, on the rice chromosomes<sup>60,93)</sup>.

*In situ* hybridization (ISH) was first reported in 1969 as a cytogenetical method to visually detect specific DNA or RNA sequences located on the chromosome, nucleus, cell, or tissue samples using a microscope. In the first report, the radio isotope (RI)-labeled ribosomal RNA was used to detect the localization of rRNA sequences on the nucleoli of the human cells<sup>28)</sup>. Since the first report ISH has been greatly improved and the method is used for the physical mapping of DNA sequences on chromosomes in both animals and plants.

In the case of the rice plant, *rbcS* gene, 28S ribosomal RNA gene, 57 kd seed protein gene, and glutelin gene, have been mapped on the chromosomes using RI-labeled probes<sup>22,90,96)</sup>. Although ISH with RI-labeled probes is very effective, the method has some shortcomings<sup>56)</sup>; (1) it takes a prolonged exposure time especially for detection of short DNA sequences; (2) silver grain signals sometimes appear at regions away from the actual gene locus and thus the resolution of the signals is not always high; (3) it needs special care and experimental instruments to handle radioactive substances.

Non-RI ISH method was developed using biotin labeling in 1981<sup>52</sup>). The modern ISH, especially fluorescence *in situ* hybridization (FISH) method makes it possible to attain high sensitivity, short detection time, and easy handling of samples. Using the FISH method in the human genome, several cloned DNA sequences, like highly repetitive DNAs, e. g., Alu, LINE sequences<sup>43,50</sup>, DNA fragments from YACs (yeast artificial chromosomes)<sup>11</sup>) and clones from cosmid libraries<sup>55,62</sup>) have been physically localized on specific chromosomes.

In plant the FISH method has also been applied to wheat<sup>69</sup>), barley<sup>54</sup>), rye<sup>1</sup>) and tobacco<sup>45</sup>). The method has been used to characterize alien chromosomes and chromosome segments<sup>31</sup>) and to elucidate the genomic constitution of allopolyploids<sup>3,80</sup>). It was also applied to detect chromosome behaviors and to map physically the genes<sup>32</sup>). In the case of rice, chromosomes are very small and the preparation of good chromosome samples is relatively difficult. Thus developing a reliable FISH method has been expected in the rice genetics and breeding researches.

In rice, a conventional genetic linkage map based on the recombination values has already been constructed<sup>49</sup>). The RFLP map based on molecular makers is now being developed<sup>60</sup>), and the number of newly cloned genes and DNA sequences is rapidly increasing. However, their positions on the chromosomes and the process of their replication have not yet been analyzed. To localize the genes in rice chromosomes and to understand the process of their replication, FISH will be one of the most important methods in the near future.

For example, detection of the centromeric regions on the chromosomes, and the assignment of genes to a respective short or long arm of a chromosome are the most important for orientation of linkage maps. Furthermore, the FISH method applied to the nuclei in the S phase of mitotic chromosome presents essential information on the replication of the genes<sup>79</sup>).

Deshpande and Ranjekar estimated that the rice genome consists of approximately 50% repeated DNA sequences<sup>8</sup>). Information on the distribution of genes and repeated DNAs sequences is important for understanding rice genome organization. The biological meaning of repeated DNA sequences in rice genome is also an important subject to be solved.

Moreover, the differences of the gene arrangements in chromosomes, which could not be revealed by the conventional and morphological observation using optical microscopes, will be clearly detected by the FISH method. The FISH method will be a powerful technique for new karyotype analysis.

In the first chapter of thesis, several experiments are conducted for the purpose to develop the FISH method which can locate genes and DNA sequences on specific chromosome regions in rice. The results obtained show that the FISH method is one of the key techniques in helping to determine the gene order and the chromosomal organization. Described in the following chapters are : (II) Development of an improved FISH method for detecting the highly repeated DNA

sequences of ribosomal RNA genes, (III) Physical mapping of the moderately repeated DNA sequences and (IV) Characterization of chromosomes from African cultivated rice (*O. glaberrima*) chromosomes using the multicolor FISH (McFISH), and (V) Conclusion.

## II. DEVELOPMENT OF FISH METHOD AND MAPPING OF 45S rDNA LOCI

### A. Introduction

Rice chromosomes involving a large 17S-5.8S-25S ribosomal RNA gene (45S rDNA) have been identified as satellite chromosomes by microscopical observation<sup>17</sup>. They are also recognized as the chromosomes with the nucleolar organizing regions (NOR chromosomes). In cultivated rice, *Oryza sativa* L. ssp. *japonica*, a pair of NOR chromosomes was detected<sup>17,51</sup>. This NOR chromosome was designated as chromosome 11<sup>17</sup> which is chromosome 9 according to the new system<sup>18,46</sup>. In *O. sativa* ssp. *indica*, two pairs of NOR chromosomes have been observed<sup>97</sup>.

Although chromosomes containing 45S rDNA show conspicuous characteristics such as satellite chromosomes, they are sometimes difficult to be identified morphologically when the copy numbers of the 45S rDNA units at a locus are small<sup>53</sup>. The *in situ* hybridization (ISH) method<sup>2,35,81</sup> offers a way out of this problem since it is based on the detection of the 45S rDNA loci was identified on chromosome 9 in *japonica* rice<sup>16,22,37</sup> while two 45S rDNA loci were detected in *indica* rice<sup>5,39</sup>. Although ISH is now widely employed in cytogenetical analysis, it is time consuming and strict experimental protocols are needed for its success. Therefore the objectives of this study were to develop a convenient and reproducible fluorescence ISH (FISH) technique in rice chromosomes for cytogenetical analysis.

### B. Materials and methods

#### 1. Plant materials and cytological procedures

Nine rice species (Table 1) were obtained either from the gene bank of the National Institute of Genetics (Mishima 411, Japan) or Hokuriku National Agricultural Experiment Station (Joetsu 943-01, Japan). Seeds of primary 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 one week before examination.

The procedures for sample preparation followed the protocol described by Fukui and Iijima<sup>17,18</sup> with minor modifications as follows : (1) the glass slides

**Table 1.** Number of rDNA loci detected in cultivated rice (*O. sativa*) and the wild species studied

Species	Genome	Varietal group	Variety name	Source* and Origin	Number of rDNA loci
<i>O. sativa</i>	AA	<i>japonica</i>	Nipponbare	HNAES, Japan	1
			Aikoku	HNAES, Japan	1
			Tushimaakamai	HNAES, Japan	1
			Tarizaohsen	HNAES, China	1
			Kouketsumochi	HNAES, China	2
			Ch78	NIG, China	1
			Ch79	NIG, China	1
	<i>indica</i>	Chinsurah BoroII	HSAES, India	2	
		Kasalath	HNAES, India	2	
		IR36	HNAES, India	2	
	<i>javanica</i>	Ketan Nanga	HNAES, Indonesia	2	
		Inakupa	NIG(221), Philippines	2	
	<i>O. rufipogon</i>	AA	annual type	NIG(W0106), India	2
perennial type			NIG(W0149), India	2	
perennial type			NIG(1944), China	1	
<i>O. glumaepathla</i>	A <sup>sp</sup> A <sup>sp</sup>		NIG(W1192), Brazil	1	
<i>O. punctata</i>	BB		NIG(W1582), Chad	3	
<i>O. officinalis</i>	CC		NIG(W0002), Thailand	3	
<i>O. eichingeri</i>	CC		NIG(W1521), Uganda	2	
<i>O. australiensis</i>	EE		NIG(W1538), Australia	2	
<i>O. brachyantha</i>	FF		NIG(W1401), Sierra Leone	1	
<i>O. latifolia</i>	CCDD		NIG(W0019), Unknown	5	

\* HNAES=Hokuriku National Agricultural Experiment Station.

NIG=National Institute of Genetics.

Figures in parentheses indicate the accession number.

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 decompressed chamber before maceration and ; (4) the chromosome preparations were flamedried.

## 2. 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., 1.5% Macerozyme R-200, Yakult Honsha, Tokyo and 0.3% Petolyase Y-23, Seishin pharmaceutical Ltd., Tokyo, 1 mM EDTA, pH 4.2) in  $2\times$ 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 five min. They were completely dehydrated through a 70/95/99% ethanol series for 10 min each and were then air-dried. Finally, they were treated with 100  $\mu$ g/ml RNase A (Sigma) in  $2\times$ SSC at 37°C for 60 min. The 45S rDNA probe<sup>87</sup> 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 (17S-5.8S-25S rDNA) and the flanking spacer regions. The probe was labeled by a random primer labeling method (Nippongene Ltd.) with biotin-dUTP following the supplier's instructions. A 15  $\mu$ l aliquot of the hybridization mixture containing 100 ng of biotinylated-45S rDNA in 50% formamide/ $2\times$ SSC was placed onto a glass slide. The solution was covered with a cover slip, sealed with liquid arabian gum and then air-dried. The glass slide with a sealed cover slip was placed on a thermal cycler (PHC-3', Techne, Cambridge, UK) that had been modified by adding an 80  $\times$  120 mm castaluminum flat plate. The programmed heating sequence was 70°C for six min and 37°C overnight. The cover slips were removed and the slides were washed with  $2\times$ SSC three times and once with  $4\times$ SSC at 37°C for 10 min each. A 70  $\mu$ l 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  $\mu$ l of 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 three times washing with BT buffer at 40°C for 5 min each, a 70  $\mu$ l of 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 propidiumiodide (PI) solution (12.5  $\mu$ g/ml in phosphate buffer, pH 6.8, 12.5  $\mu$ g/ml p-phenyldiamine dihydrochloride, with 90% glycerol) and were then examined by fluorescence microscopy.

## 3. 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 a  $512 \times 512$  pixel matrix with 256 steps of grey value for each pixel as in the images previously analyzed and reported by using the chromosome image analyzing system, CHIAS<sup>13,14,15,20</sup>. 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<sup>17,21</sup>. Chromosomal areas and 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 the computer imaging. The proper look-up-table was developed by trial and error repeatedly comparing the original microscopic images with the computer-generated images<sup>19,23</sup>. 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).

### C. Results

The G-light excitation image of the chromosomes of *O. sativa*, ssp. *indica*, cv. IR36, is shown (Plate 1a). Two pairs of fluorescent signals were observed in B light (Plate 1b). Plate 1c shows the integrated 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 in the chromosomes, an integrated image was markedly improved by image processing as shown in Plate 1c. The four signal positions of IR36 were precisely determined by using the integrated image compared with the two original images.

Eight representative examples of signal occurrence on the chromosomes found in nine *Oryza* species with six different genomes are shown (Plate 2). The 45S rDNA sites varied from one pair (Plate 2a, g), two pairs (Plate 2b, e, f), three pairs (Plate 2c, d) and five pairs (Plate 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 *Oryza* species, giving four signals as demonstrated in Plate 2e and f. Two large-, two medium- and two small-sized signals were observed among the species with six signals, although the differences between the large- and medium-sized signals are not always clear (Plate 2c and d).

On occasion the secondary constriction, the adjoining part of the chromosome and the satellite were all fluorescent (Plate 2e). Such signals were sometimes counted as two for one 45S rDNA site, so that the signal number was sometimes three, four or even six (Plate 2e). It was, however, possible to distinguish two genuine signals where two signals were noted at one site, using the chromosomal morphology observed in G excitation light. The secondary constrictions were also sometimes fluorescent (Plate 2e).

A summary of the number of 45S rDNA loci detected by the FISH method in the nine *Oryza* species with the A, A<sup>sp</sup>, B, C, E, F and CD genomes is given (Table 1). One or two 45S rDNA loci were identified in the species with the A genome. One B genome species showed three 45S rDNA loci. Two C genome species showed either two or three 45S rDNA loci. Species having the E and F genomes exhibited two and one 45S rDNA loci, respectively. The number of 45S 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 45S rDNA sites was thus observed between the species with different genomes, as well as between species with the same genome. Moreover, the number of 45S rDNA loci differed between the lines within species.

The results of a detailed examination of the number of 45S rDNA loci amongst the A genome species is shown in Table 1. Three *japonica* varieties, Nipponbare, Aikoku and Tushimaakamai, had one 45S rDNA locus. Three *indica* varieties, Chinsurah Boro II, Kasalath and IR36, exhibited two 45S rDNA loci. Two *javanica* rice varieties, Ketan Nanga from Indonesia and Inakupa from the Philippines, showed two 45S rDNA loci. On the other hand, *japonica* genotypes, Kouketsumochi, a glutinous rice from southern China had two 45S rDNA loci, while Tarizaohsen from China also had one locus.

*O. rufipogon* is an A genome wild species, consisting of two groups, perennial and annual<sup>177</sup>). One annual and two perennial types were examined. One or two 45S rDNA loci were observed in the perennial, and two 45S rDNA loci were detected in the annual type. Plate 3 shows the identification of the 45S rDNA chromosomes by the FISH method using two trisomic lines and their parental variety, IR24. Two large- and two small-sized signals were observed on the chromosomes of disomic IR24 (Plate 3a). Five signals were, however, discernible in both trisomic lines. All the fluorescent signals were located at the ends of the signal-tagged chromosomes. The trisomic for chromosome 9 displayed three

large and two medium (Plate 3b), whereas the trisomic for chromosome 10 showed two large- and three medium-sized signals (Plate 3c). The signal on chromosome 9 always showed larger and stronger intensity than did that of chromosome 10. For the third locus, the chromosome involved could not be definitely identified because of lack of the trisomic line for this chromosome.

#### **D. Discussion**

##### **1. The development of FISH method for rice chromosomes**

A non-radioactive labeling method with biotin was developed and widely applied to the ISH method<sup>30,67,81</sup>. The 45S rDNA loci of rice were localized using biotinylated probes<sup>16,37,39</sup>. Mainly due to the difficulty in preparing samples suitable for ISH and in 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<sup>16,22,29,90,96</sup>.

This study reports three improvements in the fluorescence ISH (FISH) procedure to obtain reproducible clear signals on rice chromosomes. Firstly, post-treatments were introduced to avoid the thin fluorescent covering all the field after FISH treatment, which was often observed especially under B excitation light. The four new 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<sup>88</sup>, by RNase treatment.

Secondly, a modified thermal cycler was employed throughout the denaturation process of the chromosome 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 temperature 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<sup>13,14,15,20</sup>). Image analysis for chromosome research has been effective in the identification and characterization of rice chromosomes<sup>17,18,37</sup>).

## 2. Evolutionary significance of the variability in the number of 45S rDNA loci

This study has revealed variability in the number of 45S rDNA loci among the eight diploid and the one tetraploid species within the genus *Oryza*. Such variability is rare in *Hordeum* and *Triticum*<sup>9,68</sup>), where 45S rDNA sites were investigated using the ISH technique. It is worth noting that wild species of *Oryza* have a wide range of variability as regards the number of 45S rDNA loci. Thus *O. rufipogon*, a putative ancestor of cultivated rice, has 45S rDNA variability which is similar to that of cultivated rice. Cultivated rice has either one or two 45S rDNA loci. The varieties in temperate regions have one 45S rDNA locus while those in tropical and sub-tropical regions have two 45S rDNA loci.

The *javanica* group of *O. sativa* is sometimes referred to as a tropical *japonica* since there are evidences that this subspecies is similar to the temperate *japonica* group based on RFLP analysis<sup>44,95</sup>) as well as morphological similarities<sup>75,89</sup>). The two *javanica* varieties used in this study showed two pairs of 45S 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 *Oryza* species with one NOR had already been determined to be chromosome 9 on the basis of morphology<sup>17,18,51</sup>) and the presence of 45S rDNA locus using the ISH method<sup>14,16,37</sup>). Another NOR chromosome was identified as chromosome 10<sup>39</sup>). Using the trisomic lines for chromosome 9 and 10, the signal intensity for each locus was found to be different. The locus on chromosome 9 exhibited a stronger signal intensity compared to that on chromosome 10. The third 45S rDNA locus has the weakest signal among the three 45S rDNA loci. Since there are no trisomic lines having the third locus in wild species of *Oryza*, it is difficult to identify the chromosome bearing this 45S rDNA loci. The chromosome on which this 45S rDNA locus is found is suspected to be situated on the chromosome 11, due to the conspicuous secondary constriction in the interstitial region of its long arm<sup>17</sup>). 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 species (i.e., *O. punctata* and *O. officinalis*) lends support to this hypothesis.

### III. DETECTION OF TANDEM REPEAT SEQUENCES A (TrsA) BY IMPROVED FISH METHOD

#### A. Introduction

In order to evaluate the effectiveness of the FISH method developed in the Chapter II, rice DNA sequences with fewer nucleotides were employed as the probe of FISH. The probe used in the experiment was tandem repeat sequences isolated from *O. sativa*. The genomes of higher plants contain tandem repeat sequences families. In rice, one family of tandem repeat sequences, which consists of a 360 bp sequence unit, has been identified in various cultivars of *O. sativa*<sup>7,74,99</sup>. The tandem repeat sequences (here called TrsA) are localized at the two loci, *trsA1* and *trsA2*, which show sequence divergence among varieties in genus *Oryza*<sup>73</sup>. Another locus that includes TrsA was found in *japonica* and *indica* varieties, Nipponbare and IR36, and was named as *trsA3*. The nucleotide sequences along with the flanking chromosomal sequences have been reported in *O. sativa*<sup>74,101</sup>. An *in situ* hybridization analysis has indicated that the TrsA sequences are located at the telomeric regions in long or short arms of chromosomes<sup>98</sup>. Using the polymerase chain reaction (PCR), it has been demonstrated that the TrsA sequences at *trsA1* locus, in Nipponbare, and those at *trsA2* locus, in IR36, an *indica* variety, are flanked by direct repeats of a different chromosomal sequence<sup>73</sup>. These results might suggest that the TrsA sequences were inserted by transposition, which is accompanied by duplication of the chromosomal sequences. The TrsA sequences were present at *trsA1* not only A genome in cultivated rice, but also in the lines belonging to other species in the genus *Oryza*, whereas TrsA at *trsA2* was only present in the *indica* variety, IR36.

Here, the results of physically mapping TrsA and of the nucleotide sequence analysis on flanking chromosomal sequences of TrsA at two loci, *trsA1* and *trsA3* in *O. sativa* and *O. glaberrima* are reported.

#### B. Materials and methods

##### 1. Plant materials and DNA extraction

The tandem repeat DNA sequences (TrsA, 355bp unit)<sup>74</sup> were obtained by digestion of the total genomic DNA of *O. sativa* L. cv. C5924 with *EcoRI*. The plant materials used are summarized in Table 2. They were kindly supplied by the National Institute of Genetics and National Agricultural Research Center (Tsukuba 305, Japan).

##### 2. Southern hybridization, PCR amplification and DNA sequencing

The total genomic DNA was extracted from green leaves by the CTAB method<sup>70</sup>. The isolated DNA was digested with four restriction enzymes (*BglII*, *BamHI*, *EcoRV* and *HindIII*). Southern blot analysis was carried out using non-radioactive method (ECL system, Amersham Life Science). The relative

**Table 2.** Cultivated rice (*O. sativa*) and the wild species used in this study

Species	Genome	Varietal group	Variety name	Source*	Lane No. in Fig. 3
<i>O. sativa</i>	AA	<i>japonica</i>	Nipponbare	HNAES	1
		<i>indica</i>	Kasalath	HNAES	2
<i>O. rufipogon</i>	AA			NIG(W0106)	3
				NIG(W0120)	4
				NIG(W0149)	5
				NIG(W1460)	6
<i>O. barthii</i>	A <sup>s</sup> A <sup>s</sup>			NIG(W1581)	7
<i>O. glumaepathla</i>	A <sup>sp</sup> A <sup>sp</sup>			NIG(W1192)	8
<i>O. meridionalis</i>	A <sup>m</sup> A <sup>m</sup>			NIG(W1625)	9
<i>O. punctata</i>	BB			NIG(W1582)	10
<i>O. officinalis</i>	CC			NIG(W0002)	11
				NARC(YA-12)	12
				NIG(W1521)	13
<i>O. eichingeri</i>	CC			NARC(YA-11)	14
				NIG(W1538)	15
<i>O. australiensis</i>	EE			NARC(YA-7)	16
				NIG(W1401)	17

\*HNAES=Hokuriku National Agricultural Experiment Station.

NIG=National Institute of Genetics.

NARC=National Agricultural Research Center.

Figures in parentheses indicate the accession number.

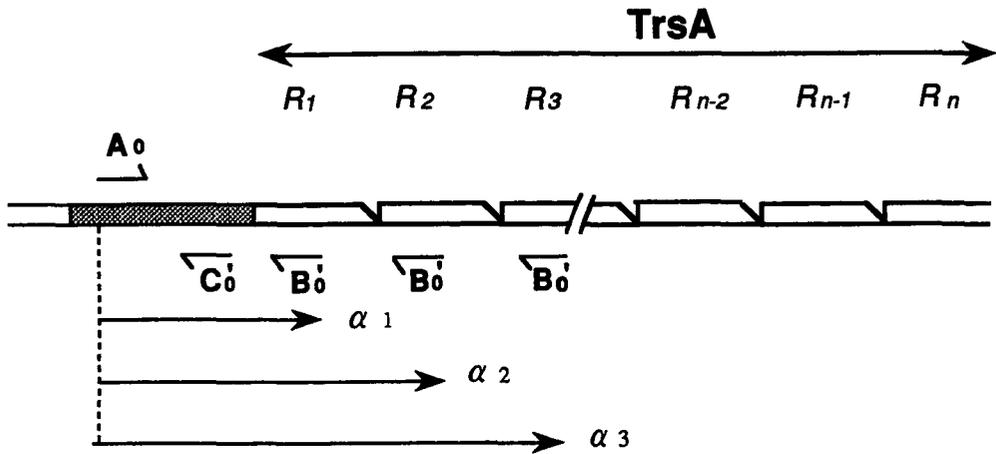
copy number of TrsA was determined by dot-blot hybridization for the nine species examined.

PCR was carried out by the standard method<sup>82)</sup> using 2.5 units of Taq DNA polymerase (Promega), 0.5  $\mu$ g of total rice DNA, and 1  $\mu$ M each for a given pair of primers. The conditions of PCR amplification have been described previously<sup>61)</sup>. Primers used are listed (Table 3).

DNA sequencing was carried out by the dideoxynucleotide chain termination method<sup>59,84)</sup>. A BcaBEST dideoxy sequencing kit (Takara) or Sequenase version. 2.0 (United States Biochemicals) was used.

The synthetic oligodeoxyribonucleotide primer, Co'-1 or Co'-2 (see Figs. 1 and 2), was labeled with <sup>32</sup>P at its 5' end using polynucleotide kinase (Takara) and used as probe for the detection of the  $\alpha$  fragments (see Fig. 2). Southern hybridization was carried out, as described previously<sup>94)</sup>, except that the temperature used for hybridization and washing of filters was 50°C.





**Fig. 2.** Schematic representation of the analysis of a junction region of *TrsA* with a chromosomal sequence by PCR. The structural features of the junction region are explained in Fig. 6.  $R_1$ ,  $R_2$ ,  $R_3$ , ...  $R_n$  are the repeating *TrsA* sequences. Primers ( $A_0$ ,  $C_0'$  and  $B_0'$ ) were used to amplify the fragments ( $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ ) containing the junction as indicated.

### 3. Chromosome sample preparation for FISH

A haploid of the cultivar *O. sativa* ssp. *japonica* cv. Koshihikari and kernels of *O. glaberrima* (W025, kindly supplied by National Institute of Genetics) were grown in a greenhouse for root tip collection. Seeds of Nipponbare and IR36 were germinated on the moist filter paper in the Petri dishes for 2–3 days. Root tips of about 1 cm in length were excised and fixed in a fixative (ethanol : acetic acid, 1 : 1) without any pretreatment.

Chromosome samples were prepared by an enzymatic maceration and air drying method<sup>17,18,36)</sup> with the following minor modifications. The root tips were subjected to decompression treatment in a modified enzymatic mixture. The composition of the mixture was 1% Cellulase Onozuka RS, 0.75% Pectolyase Y-23, 0.15% Macerozyme R200, 0.5 mM EDTA, and pH 4.2. Enzymatic maceration was carried out in a 1.5 ml microtube. The chromosome samples were stained with a 2% Giemsa solution (pH 6.8) for 15 min.

Good chromosome samples at prometaphase stages were examined and microphotographed (Axiophot, Zeiss). All the prometaphase chromosomes were identified under the microscope prior to FISH.

#### 4. Direct labeling of repeated sequence and FISH

The biotin-labeled TrsA probes were prepared by the direct labeling method. The standard PCR method performed using the thermal cycler (Perkin Elmer Cetus) according to the manufacturer's instructions. The template for the direct labeling was the plasmid pHOC154 containing an *EcoRI* fragment of the repeated sequences which had been cloned into the *EcoRI* site of pUC19 (kindly supplied by Dr. H. Ohtsubo, Tokyo University). The oligonucleotides M13/RV and M13/M4 (Nippon gene) were used as primers of direct labeling. Thirty cycles were used for PCR amplification of the TrsA and simultaneous labeling the TrsA with biotin-16-dUTP (Boehringer Mannheim Biochemicals).

The improved FISH method for signal detection in rice chromosomes described in Chapter II was used.

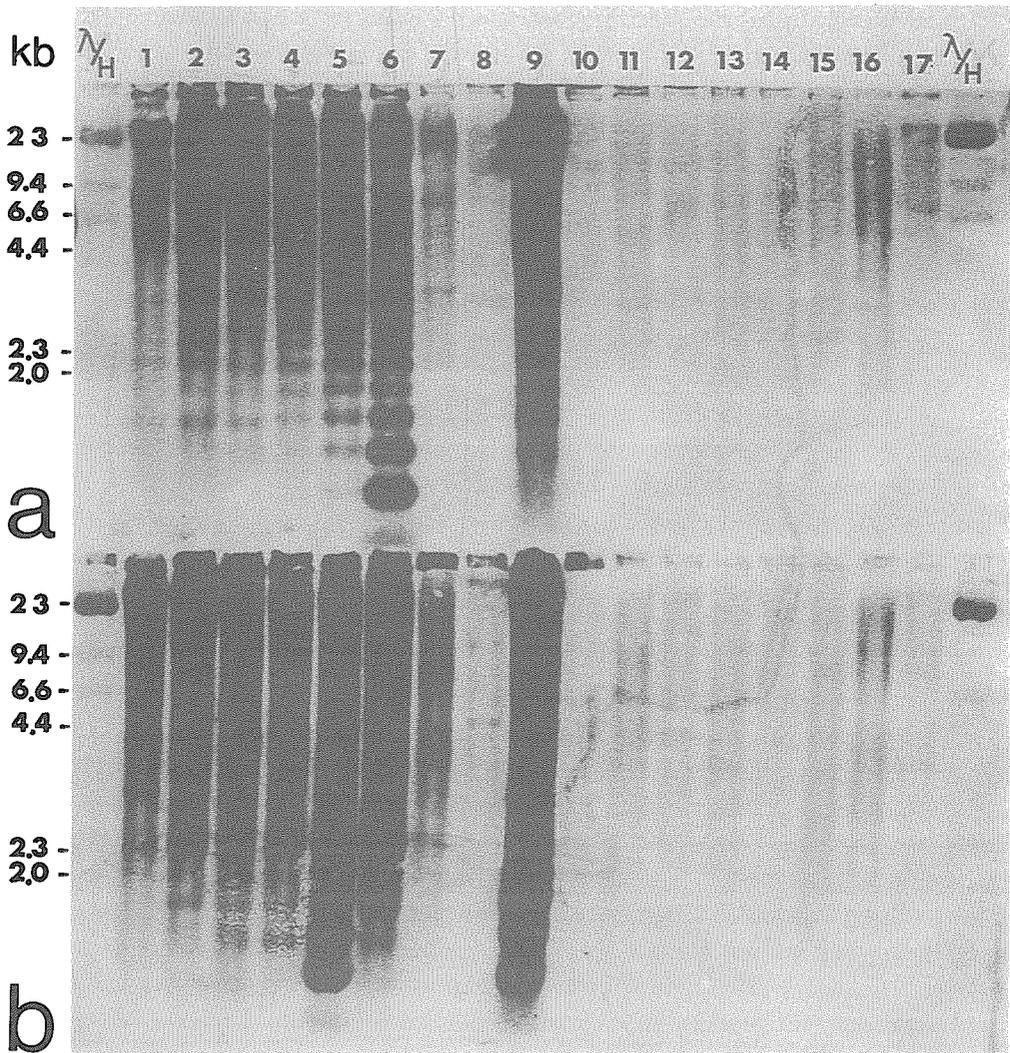
Yellowish fluorescent signals of FITC, and reddish fluorescent PI were discernible using the fluorescence microscope with the different filter sets (B10, G15, Axiophot, Zeiss) through a high sensitive color CCD camera (HCC-3600P, Floubel). After digital enhancement of the signals the two fluorescent images were integrated into a single image and pseudo-colored to reproduce the actual fluorescent colors by a look-up-table<sup>18,23</sup>.

### C. Results

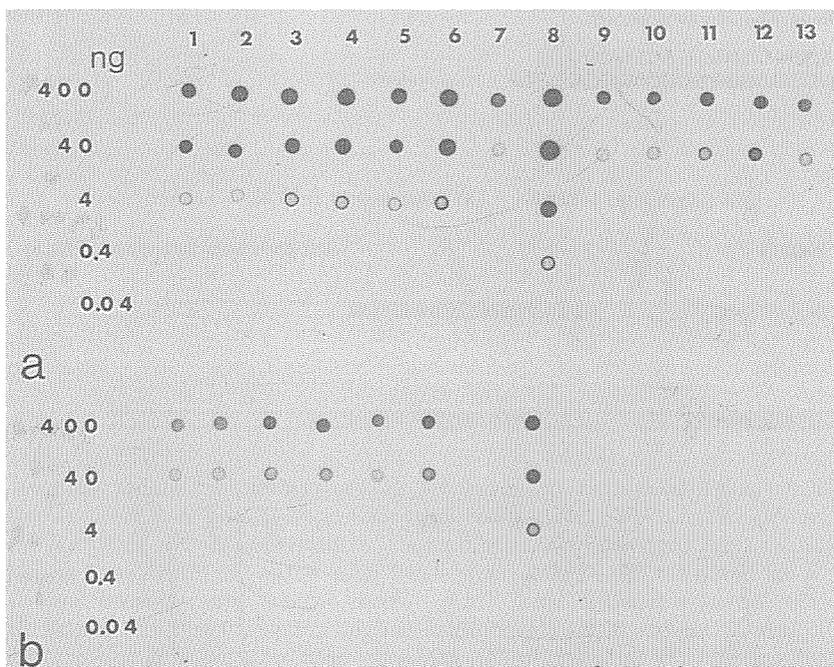
#### 1. Presence of tandem repeat sequences in genus *Oryza*

The hybridization patterns of ten *Oryza* species are shown (Fig. 3). The hybridization patterns of the DNA digested with *BglII* (Fig. 3a) and *BamHI* (Fig. 3b) were detected using Southern blot analysis with TrsA as the probe. The lanes of *O. sativa* (Lanes 1 and 2) and *O. rufipogon* (Lanes 3-6) showed the ladder formation of the signal. The ladder patterns detected in Lanes 1-6 showed the tandem distributions of TrsA sequences in rice genome. The intensity of the Southern hybridization signals was different between *O. sativa* (Lane 1) and *O. rufipogon* (Lanes 3-6). Divided band patterns among the varieties were observed in *O. rufipogon*. The three species with modified A genome showed weaker and/or smear banding pattern. Most of wild species showed weakly hybridized.

The results of dot hybridization of the genomic DNA from the nine *Oryza* species diluted serially with TrsA probe are shown (Fig. 4). Signal intensity of *O. sativa* and *O. rufipogon* was approximately the same when equivalent concentration of the probe DNA was applied. The intensities in *O. glumaepatula* ( $A^{sp}A^{sp}$ , Lane 7) and *O. meridionalis* ( $A^m A^m$ , Lane 8) differed considerably from one another. The DNA of *O. meridionalis* having a modified A genome showed much stronger signal intensity than that of *O. glumaepatula* ( $A^{sp}A^{sp}$ ) DNA. *O. glumaepatula* hybridized least with the probe among A genome species. Other *Oryza* species also showed weak hybridization. These results demonstrated that the TrsA sequences are present not only in lines with A genome, but also in those with other genomes with repeated numbers lower than that in A genome.



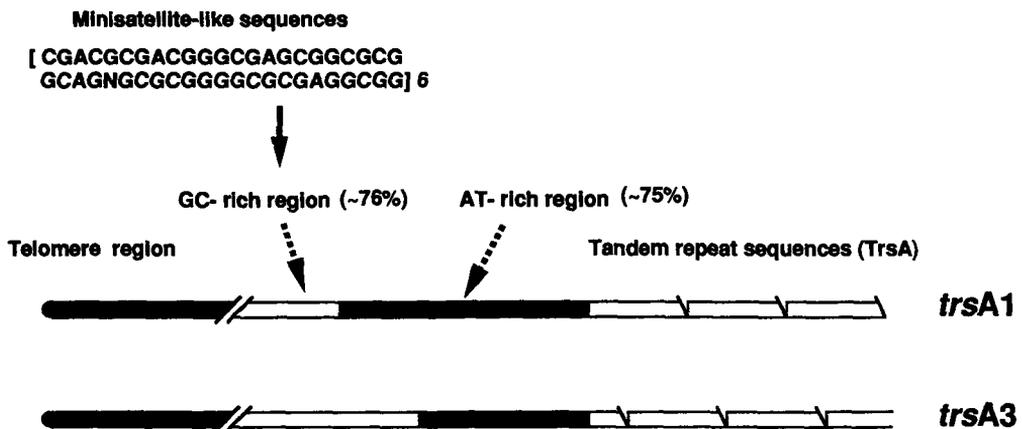
**Fig. 3.** Southern hybridization with TrsA. The total DNA from wild species was digested with restriction enzymes (*Bgl* II, Fig. 3a and *Bam* HI, Fig. 3b), fractionated electrophoretically on an 0.8% agarose gel, transferred to a Nylon filter, and then hybridized to TrsA. Lane 1 ; *O. sativa*, Nipponbare, Lane 2 ; Kasalath, Lanes 3-6 ; *O. rufipogon*, Lane 7 ; *O. barthii*, Lane 8 ; *O. glumaepatula*, Lane 9 ; *O. meridionalis*, Lane 10 ; *O. punctata*, Lanes 11 and 12 ; *O. officinalis*, Lanes 13 and 14 ; *O. eichingeri*, Lanes 15 and 16 ; *O. australiensis*, Lane 17 ; *O. brachyantha*.



**Fig. 4.** Dot-blot hybridization with TrsA under (a) low stringent and (b) high stringent conditions. Figures at the left hand side show the amount of DNA applied. Lane 1 ; *O. sativa*, cv. Nipponbare, Lane 2 ; *O. rufipogon* (W0120), Lane 3 ; *O. rufipogon* (W0149), Lane 4 ; *O. rufipogon* (W1460), Lane 5 ; *O. rufipogon* (YA-2), Lane 6 ; *O. rufipogon* (YA-4), Lane 7 ; *O. glumaepatula*, Lane 8 ; *O. meridionalis*, Lane 9 ; *O. punctata*, Lane 10 ; *O. officinalis*, Lane 11 ; *O. eichingeri*, Lane 12 ; *O. australiensis*, Lane 13 ; *O. brachyantha*.

## 2. The flanking TrsA chromosomal sequences

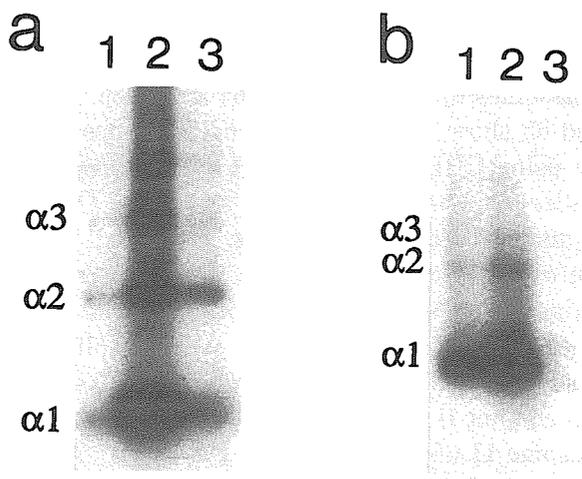
Ohtsubo et al. (1991) has isolated several phage  $\lambda$  clones with TrsA sequences, which are related to the flanking chromosomal sequence in *O. sativa* cv. Nipponbare. Figure 5 shows a schematic representation of the structures of the DNA segments that contain the junction regions with TrsA at two loci, *trsA1* and *trsA3*, which were present in two phage  $\lambda$  clones. Nucleotide sequences of the flanking chromosomal regions of TrsA were determined (Fig. 1). There existed a GC-rich sequence in the region extending from the AT-rich sequence that was joined with the TrsA sequences at each locus (Figs. 5 and 1). A GC-rich sequence has also been observed in the flanking region of TrsA at another locus<sup>101</sup>). In the GC-rich sequence in the flanking chromosomal sequence at *trsA1*, there existed obvious short repeated sequences (Fig. 1), which resemble the minisatellite sequences rich in GC in the subtelomeric regions of chromosomes in the animal cells (see Fig. 5). This suggests that the TrsA sequences are located at the subtelomeric regions.



**Fig. 5.** Schematic representation of structures of the regions containing tandem repeat sequences, TrsA. The two regions (here called *trsA1* and *trsA3* loci) contain the repeated TrsA sequences (open thick arrows) and the flanking chromosomal sequences (stippled boxes for the At-rich regions and open boxes for the GC-rich regions) that appear at each junction with TrsA. These regions are supposed to be in the subtelomeric regions of the chromosome and are connected with the telomere regions (solid boxes). The minisatellite-like sequences seen in the GC-rich region at the *trsA1* locus is shown.

### 3. Presence of TrsA at two loci in rice lines with A genome

A simple method has previously been developed to identify and characterize the fragments containing the junction of TrsA with the flanking chromosomal sequences by PCR using a pair of synthetic oligonucleotide primers (Bo' and Ao). The primers were found to hybridize a sequence inside TrsA and a flanking chromosomal sequence, respectively (Fig. 1). PCR would generate the amplified fragments forming a ladder of bands differing in size by 360 bp ( $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  in Fig. 2), when tandem repeats of TrsA are connected with the chromosomal sequence at a given locus<sup>73</sup>). This method was applied to determine the presence of the TrsA sequences at *trsA1* and *trsA3* in IR36, *O. glaberrima* GMS1 and Nipponbare. As shown in Fig. 6a, PCR using primers Ao-1 and Bo'-1 generated fragments forming a ladder of bands from the total DNA prepared from the three rice lines examined. This confirms that the TrsA sequences are present at the *trsA1* locus in all these rice lines. PCR using primers Ao-2 and Bo'-2 generated fragments forming a ladder of bands from total DNA prepared from two different *O. sativa* lines, but did not from *O. glaberrima*, GMS1 (Fig. 6b). This indicates that the TrsA sequences are present at the *trsA3* locus in the *O. sativa* lines, but not in *O. glaberrima*, GMS1.



**Fig. 6.** Agarose (1.8%) gels showing the fragments amplified by PCR. Fragments ( $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$ ; see Fig. 2) containing the junction between a chromosomal sequence and TrsA at *trsA1* (a) and at *trsA3* (b) were visualized by autoradiography after Southern hybridization analysis using the  $^{32}\text{P}$ -labeled Co' primer (see Table 3 and Figs. 1 and 2). Samples in lanes 1, 2 and 3 in each panel are those amplified by PCR using the total DNA prepared from *O. sativa* cv. IR36, *O. sativa* cv. Nipponbare and *O. glaberrima*, GMS1 as templates, respectively.

#### 4. Identification of the chromosomal locations of TrsA by FISH

Physical mapping of TrsA was carried out, using the improved FISH method and somatic prometaphase chromosomes. Plates 4-6 show the somatic prometaphase chromosome complements.

Plates 4a, 5a and 6a show Giemsa-stained chromosomes of haploid Koshihikari derived from anther culture, IR36 and *O. glaberrima* (W025). The signal locations of TrsA were checked on chromosome spreads in the three lines whose chromosome numbers had been identified prior to using FISH method. Condensation pattern or uneven condensation was characteristic to all of the chromosomes and each chromosome could be identified based on the condensation pattern of *O. sativa* chromosomes previously obtained<sup>18,37</sup>.

Two chromosomes indicated characteristics corresponding to the chromosomes 6 and 12 (by arrowheads in Plate 4a). These chromosomes showed a clear signal on the distal end of long arm. In the case of cv. Nipponbare, two pairs of hybridization signals were detected (data not shown) in the same chromosomes.

The physical mapping of the twelve signals on the chromosomes of IR36 is shown (Plate 5a and 5b). By using the Giemsa-stained image, all the chromosomes were identified (Plate 5a). The chromosomes where TrsA is located were determined to be chromosomes 5, 7, 8, 9, 10 and 11. A clear fluorescent signal

was detected at the distal end of the long arm of all the chromosomes by FISH (Plate 5b). In none of the cases hybridization signals were located on the short arm of the chromosomes in both Koshihikari and IR36.

Plates 6a and 6b show *O. glaberrima* chromosomes Giemsa-stained and an integrated image using CHIAS2 after FISH. The three pairs of hybridization signals were localized on chromosomes 5, 6, and 7. In *O. glaberrima*, the signals were also at the distal end of the long arms.

An ideogram of the results obtained in this chapter for *O. sativa* is shown (Fig. 7). Interestingly, all the signals were located at the subtelomeric regions on long arms of the chromosomes. The ideogram was followed by a quantitative chromosome map<sup>17)</sup>. The 17S-5.8S-25S rDNA locus was detected by the experiment described in chapter II. One 5S rDNA locus was localized in the proximal region of chromosome 11 of cv. Nipponbare<sup>42)</sup>.

#### D. Discussion

In this study, TrsA has been physically mapped on the distal end of the long arms of two to six chromosome pairs. Successful detection of a moderately repeated DNA sequence demonstrates the usefulness of FISH.

In this chapter, the junction regions containing TrsA and its flanking chromosomal sequences were analyzed by nucleotide sequencing and by the PCR. It has been shown that TrsA was present at one locus *trsA3* in both *indica* and *japonica* varieties of *O. sativa* but not in *O. glaberrima* GMS1, whereas TrsA is present at the other locus *trsA1* in all the rice lines examined. TrsA at the locus called *trsA2* has previously been shown to be present in the *indica* variety, IR36, but not in the others including the *japonica* variety Nipponbare<sup>73)</sup>. FISH analysis has demonstrated that the TrsA sequences were present at several loci. TrsA does not always exist in loci *trsA1*, *trsA2* and *trsA3*, depending on the lines. These results support the previous results which suggested that TrsA sequences are inserted in various sites via transposition during genetic differentiation of the *Oryza* species with A genome. Transposition of several units of TrsA may have occurred at various sites by a genetic mechanism involving duplication of a target sequence. The TrsA sequences, once inserted at a locus, would be subsequently amplified in tandem during replication of chromosomes, as previously discussed<sup>73,74)</sup>. It was assumed that transposition probably occurred at random sites. FISH analysis, however, demonstrated that the amplified TrsA sequences were present in the subtelomeric regions of long arms of the chromosomes, showing that the amplification is dependent on the location. This indicates that TrsA transposed to the sites in the subtelomeric regions can be amplified, but that TrsA transposed to the sites other than those in the subtelomeric regions fails to be amplified, probably due to a deleterious effect on the survival of the *Oryza* species.

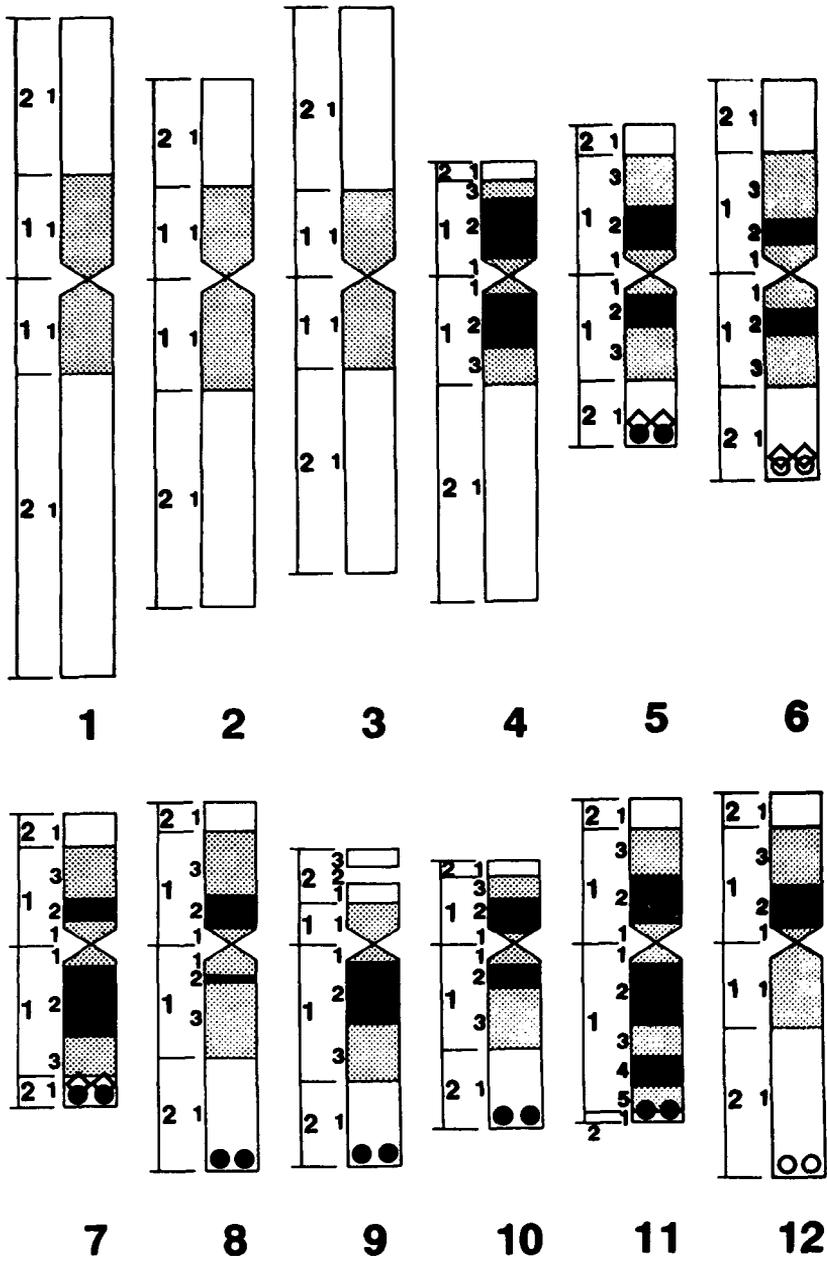


Fig. 7. Ideogram representing TrsA sites. ○ ; Koshihikari. ● ; IR36. ◇ ; *O. glaberrima*.

#### IV. CHARACTERIZATION OF *ORYZA GLABERRIMA* CHROMOSOMES BY McFISH METHODS

##### A. Introduction

*Oryza glaberrima* Steud. is an Africal cultivated rice mainly distributed in the savanna along the southern fringe of the Sahara desert<sup>78</sup>). Another cultivated rice species, *O. sativa* L., and *O. glaberrima* have been considered to have the same A genome from the cytological observation of the meiotic pairings of their F<sub>1</sub> plant<sup>63</sup>). *O. glaberrima*, endemic to West Africa and differentiated from a wild rice species, *O. barthii*<sup>6)</sup> or *O. breviligulata*<sup>66,76)</sup>, has several genes tolerant to various adverse conditions and to diseases and insects<sup>79</sup>). These characteristics might be valuable as potential genetic resources for rice breeding and even for *O. sativa*, although *O. glaberrima* itself has not been improved by modern breeding techniques<sup>78</sup>). Intensive breeding of *O. glaberrima* has begun with the establishment of the West African Rice Development Association (WARDA) in 1970.

It would be useful for effective breeding of *O. glaberrima* to characterize not only genetic traits but also cytological features of this species. Many studies have been conducted on the genetic traits of *O. glaberrima*<sup>65,86,100</sup>). Few studies, however, have been reported on detailed cytological features of *O. glaberrima*. The only paper to date reports a general similarity between the karyotypes of *O. glaberrima* and *O. sativa*<sup>33</sup>).

In this study we have reported the detailed chromosomal characteristics of *O. glaberrima* using and developing the several new technologies of image analyses<sup>14,15</sup>), micromanipulation<sup>24,25</sup>), and molecular cytology<sup>12</sup>) modified especially for the current study.

##### B. Materials and methods

###### 1. 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 a fixative (ethanol : acetic acid, 1 : 1) for at least 3 h without any pretreatment.

Chromosome samples were prepared by an enzymatic maceration/air drying method<sup>17,18,36</sup>) 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<sup>72</sup>). The chromosome samples were stained with a 2% Giemsa solution (pH 6.8) for 15 min.

Chromosome samples at resting, prometaphase, and metaphase stages were examined and microphotographed (Axophot, Zeiss). All the prometaphase chromosomes with clear condensation pattern (CP)<sup>26</sup>) were identified under the microscope prior to *in situ* hybridization. Five good prometaphase chromosome

complements were analyzed by imaging methods<sup>40</sup>. Two image parameters of relative length and arm ratio were measured for all the chromatids and the data were analyzed statistically. The condensation pattern (CP)<sup>26</sup> of each chromosome was also checked by imaging method<sup>71</sup>. Samples were then destained by 70% ethanol and used for further experiment.

## 2. Direct cloning and direct labeling of 17S and 5S rDNA of *O. glaberrima*

45S rDNA consists of a 17S-5.8S-25S rDNA gene cluster in rice genome, and a part of 17S rDNA gene was used for the probe to detect the 45S rDNA loci. Digoxigenin-labeled 17S rDNA (Dig-17S rDNA) and biotin-labeled 5S rDNA (Bio-5S rDNA) probes were prepared by direct cloning and direct labeling methods. Fixed root tips were subjected to enzymatic maceration/air drying on a  $\phi$ 35 mm heat absorptive, filmlined culture dish. The octagonal disks 2 mm in diameter with about one hundred rice nuclei on one slide were dissected out of the film using an argon ion microlaser beam through a 100x objective (ACAS470, Meridian). The disk was then recovered into a 0.5 ml microtube and used as a DNA template for PCR<sup>25</sup>.

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'-TTGTCACCTACCTCCCGTGT-3') and (5'-GATCCCATCAGAACTCCGAAG-3' ; 5'-CGGTGCTTTAGTGCTGGTATG-3') were used for the direct cloning and the subsequent direct labeling of respective 17S rDNA (455bp) and 5S rDNA (303bp) probes<sup>24,91</sup>. The first thirty cycles of the PCR amplified rDNAs directly from the nuclei and the second thirty cycles simultaneously amplified and labeled 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<sup>24,42</sup>.

## 3. Multicolor fluorescence *in situ* hybridization (McFISH) and image analysis

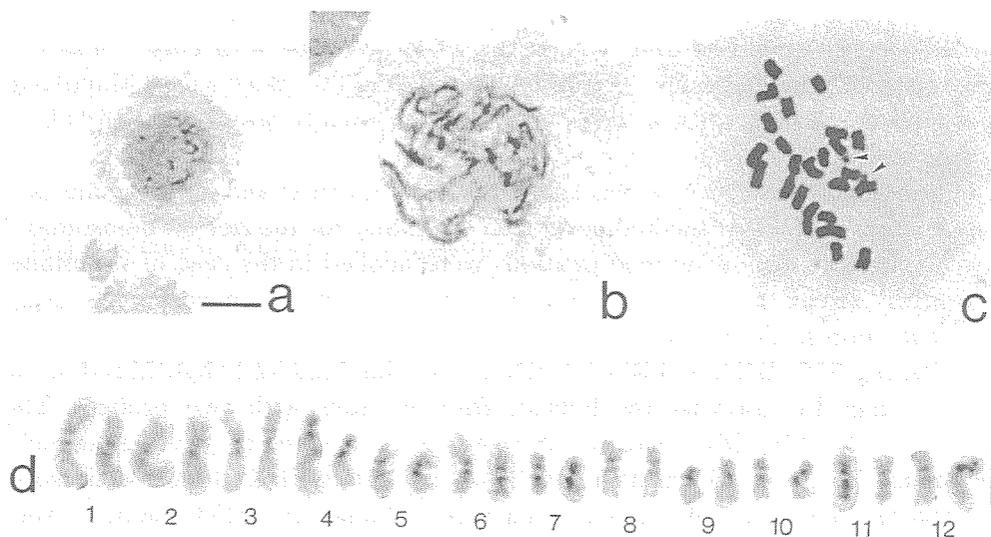
An improved FISH method developed especially for the rice chromosomes<sup>27</sup> was employed. Following modifications were applied in the case of simultaneous signal detection of the 45S and 5S rDNA loci in the chromosomes using different fluorescent colors.

The Dig-17S rDNA and Bio-5S rDNA were simultaneously hybridized to the chromosomes by applying the hybridization mixture with two probes. The hybridized Dig-17S rDNA and Bio-5S rDNA were detected by anti-digoxigenin-rhodamine (20  $\mu$ g/ml, Boehringer Manheim) and avidin-FITC (Fluorescein isothiocyanate) conjugate (20  $\mu$ g/ml, Boehringer Manheim). FITC signals were enhanced by secondary immunological reaction of the biotinylate 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, 20  $\mu$ g/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) and separately recorded in the three image frame memories (768×512 pixel matrix with 8 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.

### C. Results

The somatic chromosomes at different stages in a mitotic cell cycle are shown in Fig. 8. Figure 8a shows a nucleus at the resting stage demonstrating chromatin threads and chromomeric granules. The chromomeric granules were scattered throughout in a nucleus. The numbers of the heavily stained granules varied between the nuclei. Figure 8b and 8c 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 genus *Oryza* and other plants with small chromosomes. The metaphase chromosomes were uniformly condensed to about 2  $\mu\text{m}$  without any



**Fig. 8.** Giemsa-stained somatic chromosomes of *O. glaberrima*. a ; Chromosomes at resting stage. b ; Chromosomes at mitotic late-prophase stage. c ; Chromosomes at mitotic metaphase stage. d ; Chromosomes at prometaphase stage. Scale bars show 5  $\mu\text{m}$  for a, b and c ; and 5  $\mu\text{m}$  for d.

pretreatment. A pair of satellite chromosomes were observed as indicated by arrowheads (Fig. 8c).

Prometaphase chromosomes are arranged in the order of a new chromosome numbering system<sup>46,48</sup> in Fig. 8d. Condensation pattern (CP) or uneven condensation featured characteristics of all the chromosomes and each chromosome could be identified based on the CP revealed in the prometaphase chromosomes of *Oryza sativa*<sup>18,37</sup>.

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 4),

**Table 4.** Numerical data of somatic chromosomes of *O. glaberrima*

Chromosome No.	Relative Length (%) <sup>a)</sup>	Arm Ratio (L/S) <sup>a)</sup>	Remarks by observation <sup>b)</sup>
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 <i>O. sativa</i>
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)	1.5±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 <i>O. sativa</i>
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

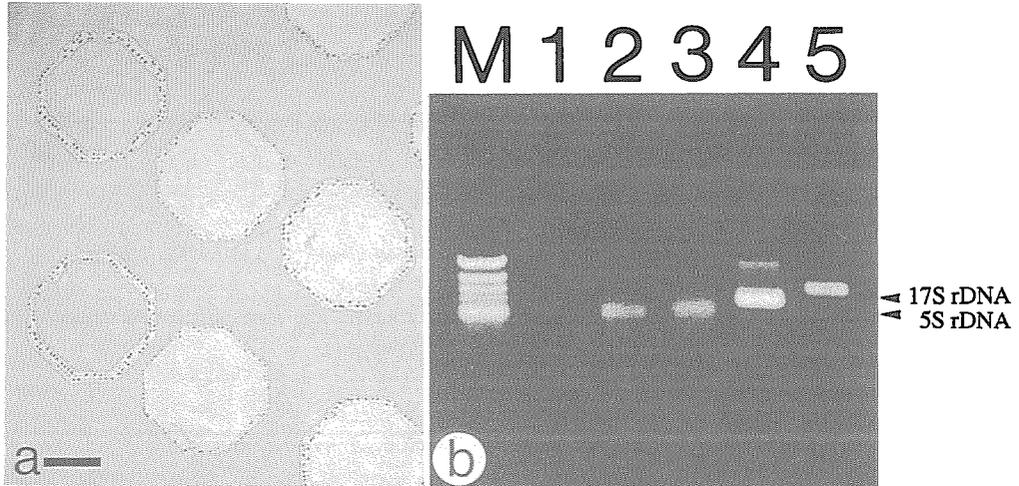
a : Mean ± s. d. Length of the satellite was excluded from the calculation. Figures in parentheses indicate the values of *O. sativa*<sup>17)</sup>

b : Chromosomes characteristics specific to *O. glaberrima*

\*\* : Significantly different between *O. glaberrima* and *O. sativa* at 1% level

although the relative length of chromosome 1 and the arm ratios of chromosome 4 and 5 were significantly different at the 1% level. Several minor modifications are also detected in the minor characteristics, which are also listed in Table 4. The FUSC (faint unstable small condensation)<sup>37</sup>, which was observed in *O. sativa* chromosomes, was also detected especially at the telomeric region of chromosomes 6 and 12 in the chromosomes of *O. glaberrima*. The satellite of chromosome 9 was much more distinct than that of *O. sativa*.

Figure 9 shows microdissection of the template disks and the agarose gel electrophoresis of the PCR products. Figure 9a demonstrates 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 recovered in a 0.5 ml microtube. The results of direct cloning and labeling are shown in Fig. 9b. Lane M is the molecular size marker ( $\phi$ X 174/*Hinc* II) 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 labeled 5S rDNAs, respectively. A retardation of the band in lane 3 indicates the incorporation of biotin to the amplified 5S rDNA. Lane 4 and 5 correspond to the direct cloning and labeling of 17S rDNA with digoxigenin. The same tendency is observed more clearly in the case of 17S rDNA with digoxigenin.



**Fig. 9.** Microdissection and direct cloning of 5S rDNA and 17S rDNA. a ; Dissection of the octagonal disks with about a hundred nuclei. Bar indicates 1 mm. b ; Direct labeling of 5S rDNA and 17S rDNA by PCR method. Agarose gel (1%) electrophoresis. Lane M ; Molecular marker,  $\phi$ X174/*Hinc* II, Lane 1 ; Negative control, Lane 2 ; 5S rDNA, Lane 3 ; 5S rDNA labeled by biotin, Lane 4 ; 17S rDNA, Lane 5 ; 17S rDNA labeled by digoxigenin.

McFISH simultaneously revealed the number of both the 5S and 45S rDNA loci using the Bio-5S rDNA and Dig-17S rDNA as the probes (Plate 7). Plates 7a, 7b and 7c illustrate the DAPI image of interphase chromosomes, the same image through a B10 filter set with a clear pair of signals of the 5S rDNA locus appearing within the complement, and the image through a G15 filter showing a pair of signals of the 45S rDNA locus within the complement, respectively. The doublet formation of the signals at the 5S rDNA locus indicates that the 5S rDNA has been replicated (Plate 7b).

*O. glaberrima* has only one locus for both the 5S and 45S rDNA as in *O. sativa*<sup>22,42</sup>. Plate 7d shows a digital integration of the three different fluorescent images. Interphase chromosomes and the 5S and 45S rDNA loci are illustrated in the different colors - blue, yellow, and red, respectively.

McFISH simultaneously revealed the number and position of both the 45S and 5S rDNA loci in the chromosomes using the Bio-5S rDNA and Dig-17S rDNA as the probes (Plate 8). Plate 8a illustrate the DAPI image of *O. glaberrima* chromosomes. All the chromosomes are readily identified, since all of them have already been identified in prior to McFISH. One pair of *O. glaberrima* chromosomes have three heavily condensed regions along the chromosome, and thus the chromosomes have been identified as the chromosome corresponding to chromosome 11 of *O. sativa* (Trimodal or Mitsuyama)<sup>37</sup>. The chromosomes showing the secondary constriction have been identified as the corresponding chromosomes to chromosome 9 of *O. sativa* (Satellite or SAT)<sup>37</sup>, since the satellites are clearly observed in the Giemsa-stained images of these chromosomes.

Plate 8b shows an integrated image of the DAPI stained chromosome image and the yellowish signal image of 5S rDNA locus. Two sites of a 5S rDNA locus are clearly observed in chromosome 11. The detailed observation proved the locus position at the proximal region of a short arm of chromosome 11. Doublet formation of the 5S rDNA signals are observed in most cases indicating the existence of the 5S rDNA locus in each replicated chromatin.

Plate 8c shows an integrated image of the DAPI stained chromosome image with the reddish signal image of a 45S rDNA locus. Two sites of a 45S rDNA locus are clearly detected at the distal end of chromosome 9. In the case of 45S rDNA, the doublet signals were not observed but the single fused signal and/or extended signal were observed at the secondary constriction, indicating the 45S rDNA locus to correspond to the nuclear organizing region. Plate 8d shows digital integration of three fluorescent images of the chromosomal and two signal images. One 5S rDNA and 45S rDNA loci are simultaneously detected on respective chromosome 11 and 9 under the current condition of McFISH.

Figure 10 depicts the locations of both the 45S and 5S rDNA loci on the quantitative idiogram of *O. sativa*<sup>17</sup>, since most of the relative lengths and arm ratios of *O. glaberrima* chromosomes were not significantly different from those of *O. sativa* as shown in Table 4. Moreover the condensation patterns of the *O.*

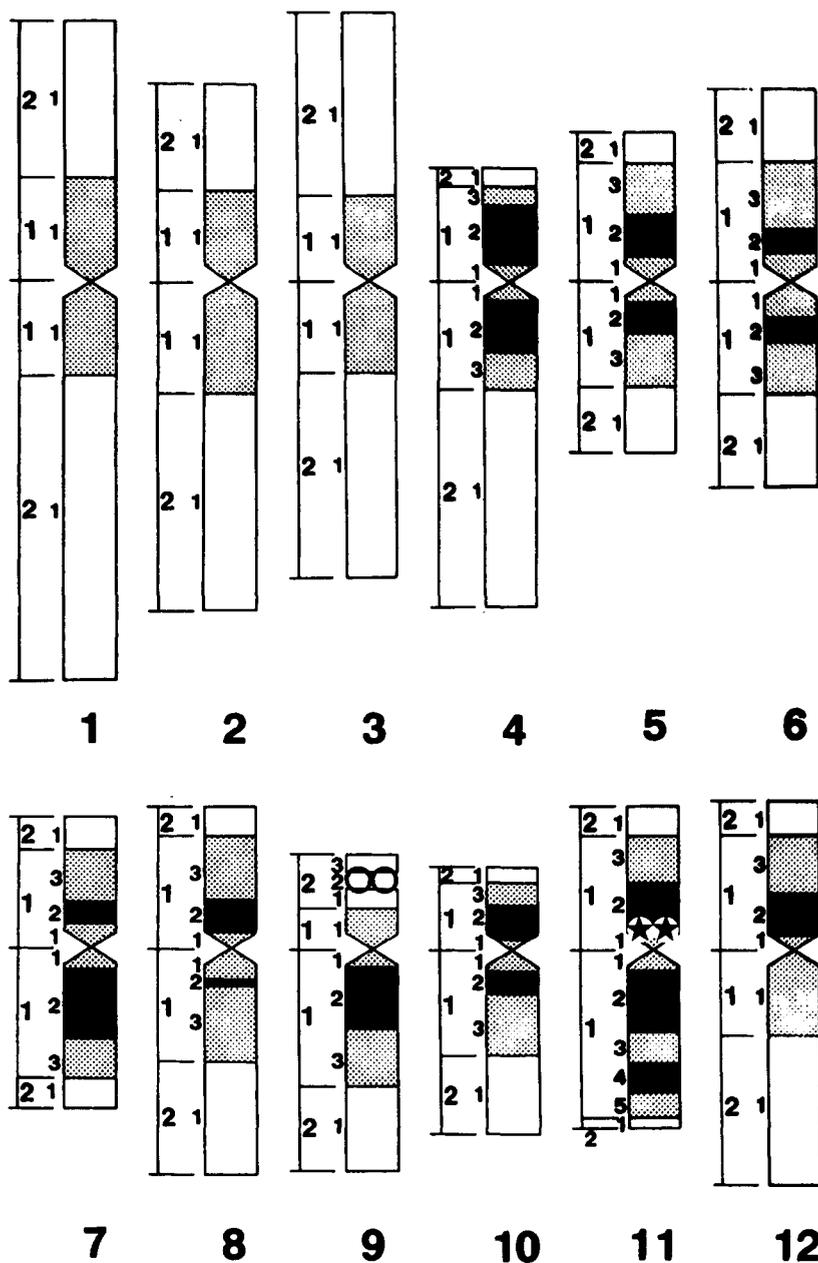


Fig. 10. Chromosome map of the 45S rDNA (O) and 5S rDNA (★) loci in *O. glaberrima*.

*glaberrima* prometaphase chromosomes were not also remarkably different from those of *O. sativa* by both visual inspection and imaging methods. The map of 45S rDNA and 5S rDNA loci demonstrates the same localization of the two ribosomal RNA gene clusters in *O. glaberrima* and *O. sativa*.

#### D. Discussion

Detailed cytological characteristics of *O. glaberrima* detected through karyotyping chromosomes and McFISH have been presented. The detailed karyotype analysis reveals that the morphology of *O. glaberrima* chromosomes is quite similar to that of *O. sativa*<sup>17,18)</sup>. The fact that *O. glaberrima* and *O. sativa* ssp. *japonica* have the same number and localization of both the 45S rDNA and 5S rDNA loci in the genome is further evidence of the essentially similar nature of the chromosome organization between the two species. The number of the 45S rDNA locus varied within the A genome species. *O. sativa* spp. *indica* and some *O. rufipogon* accessions have two 45S rDNA loci<sup>27)</sup>. The basic similarity of the karyotype of both species would be helpful to establish a complete trisomic series of *O. glaberrima*, in which a complete set of trisomic series has not developed yet<sup>38)</sup>. Similarity in the chromosome morphology has been reported using the squash method, although the resolution of chromosome morphology was limited<sup>33,34)</sup>. The normal behavior of meiotic chromosomes of the hybrids between *O. sativa* and *O. glaberrima*<sup>64)</sup> and the following genetic studies would further support the current conclusion. Several mutations in *O. glaberrima* chemically induced by EMS treatment were respectively comparable to mutations obtained in *O. sativa*<sup>85)</sup>. For example, a glutinous gene mutant appeared to be allelic with the *ux* gene of *O. sativa*. The two species are similar in genetic architecture for liguleless, long empty glume, brittle culm, and short culm and also in the results of comparative mutation research<sup>78)</sup>.

Prometaphase chromosome samples without cytoplasmic debris prepared by the enzymatic maceration/air drying method made it possible to examine minute differences in chromosomes, and some minor differences were detected between *O. glaberrima* and *O. sativa*. The advantage of the prometaphase stage for chromosome observation<sup>34)</sup> and the enzymatic maceration for chromosome preparation<sup>51)</sup> have already been pointed out. The telomeric FUSCs, which are also observed in *O. sativa* ssp. *japonica*<sup>37)</sup> are the one of the distinctive characteristics between these two species. A A genome specific repeated sequence<sup>74)</sup> hybridized *in situ* to the telomeric FUSCs and the chromosome numbers concerned were three in *O. glaberrima* and two in *O. sativa* ssp. *japonica* (Chapter III). The size of the satellite of *O. glaberrima* was also larger than that of *O. sativa*. These facts suggest that the chromosome organizations of *O. glaberrima* and *O. sativa* have been subjected to modification, especially at the distal regions of the chromosomes, during speciation.

The new technologies adopted and developed in the current study are effec-

tive in examining cytological characters and chromosome organization. The importance of the imaging method can not be overemphasized in the sciences where morphological information is indispensable. The idiogram developed by the imaging method is not only basic for the genetic and breeding research but also useful for a molecular and biochemical studies of *O. glaberrima*, presenting quantitative data on chromosomes. Microdissection for the target object is now a routine task even for the chromosomes including large-sized ones in barley to small-sized ones in rice<sup>25</sup>). A series of technologies including microdissection of a specific chromosomal region, subsequent direct cloning, and labeling of the DNA from the chromosomal fragment recovered are now available for applications in molecular cytological research<sup>24,41</sup>).

*In situ* hybridization is an effective method to physically map genes in a certain region of chromosomes<sup>12</sup>). Rice 45S rDNA loci have been localized by using the <sup>125</sup>I-labeled rRNA probe<sup>22</sup>), biotin-labeled rDNA probe<sup>16,37,39</sup>). A reproducible improved FISH for rice chromosomes has already been developed. It consists of three advances ; noise reduction by a series of post-treatments after chromosome preparation, regulation of the accurate temperature during the FISH procedures by using a modified thermal cycler, and signal enhancement and image integration by the CHIAS 2. Variability in the number of the 45S rDNA loci among the nine *Oryza* spp. has been revealed by the method<sup>38</sup>).

McFISH has succeeded in rice using directly cloned and labeled DNA probes. This opens the way not only for more effective mapping of different DNA sequences in the rice chromosomes, but also for determining order of the several clones within a chromosome<sup>62</sup>). 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 a single copy gene are also likely to be achieved in the near future.

## V. CONCLUSION

This study has been carried out to characterize the karyotype of *Oryza* species by using advanced methods of molecular cytology especially in reproducibly mapping certain DNA sequences on rice chromosomes. For this purpose, an improved fluorescence *in situ* hybridization (FISH) method has been developed. To attain this objective, three major innovations are reported. Firstly, post-treatments were developed to reduce the noise after FISH. Secondly, accurate regulation of temperature during the FISH procedure using a modified thermal cycler was employed to guarantee reproducibility. Thirdly, imaging technology was adopted to enhance the signal of FISH by constructing new software. As a result, visual detection of fluorescence from a probe DNA by FISH in rice has been achieved.

Using this FISH method, variation in the number of rDNA loci among nine

*Oryza* spp. with different genomes has clearly been detected. *Japonica* varieties from Japan or China had one 45S rDNA locus, while *indica* and *javanica* varieties had two 45S rDNA loci. The chromosomes with the 45S rDNA loci completely agreed with the results of RFLP maps<sup>57,93</sup>. Furthermore, in wild rice, the 45S rDNA loci were successfully detected and a third locus, which does not exist in cultivated rice, was detected for the first time. In *O. rufipogon*, a putative ancestor of *O. sativa*, the variability of 45S rDNA loci was recognized corresponding to the difference between *japonica* and *indica* subspecies. Therefore there is a possibility that the differentiation between *japonica* and *indica* had already existed in *O. rufipogon* before the appearance of *O. sativa* (Chapter II).

The existence of the tandem repeat sequences is essential to understand rice genome organization. TrsA (tandem repeat sequence A) is thought as a transposable element located on the three loci (*trsA1*, *trsA2* and *trsA3*) in two lines of *O. sativa* and a line of *O. glaberrima*. It was supposed to be localized at the subtelomeric sites linking with the telomeric sequences by the DNA sequence analysis. Physical mapping of the TrsA revealed that it is located in the distal ends of several chromosomes. Although the positions were different among the three lines, all of the positions were in the subtelomeric regions of the long arm of chromosomes. From these results, it is suggested that specific DNA sequences are related to the chromosome structure. Repeated sequences are the great obstacles for chromosome walking and gene tagging. Exact mapping of the repeated sequences in rice genome will provide a clue to dissolve these problems (Chapter III).

One of the significant contributions of this research to rice genetics is that a new method has been developed and that the method has opened the way to new molecular karyotyping. In addition to the ordinary karyotyping, information on the gene locations in the chromosomes by using a McFISH method, which has further been developed based on a FISH method, has revealed essential similarity of the chromosomal locations among the similar genes. Furthermore, the African cultivated rice *O. glaberrima*, in which molecular genetic research has scarcely been carried out, has been subjected to the new chromosome manipulations, e.g., direct cloning and direct labeling in order to clone the ribosomal genes. The results demonstrated also have opened the way to the gene mapping in other minor crops.

The development of a McFISH method is essential in the progress in the analysis of replication patterns of the genes and physical mapping of the genes on the same rice chromosome. Using this technique, the physical distance between two genes on the linkage map is estimated and their locations in the nucleus could be analyzed. McFISH makes it possible to physically map the genes in detail (Chapter IV).

The FISH method developed in this study has opened further directions to study in the molecular cytogenetics of rice. It will enable us to recognize the fine

structure of a chromosome. Understanding a distribution pattern of individual genes and repeated sequences will be greatly improved by analyzing the physical organization and genome dynamism of a chromosome and of a nucleus. Integration of both the results accumulated by the classical rice research and the new data obtained by the technologies recently developed will accelerate the development of rice genetics and breeding.

## VI. ABSTRACT

The development of an improved fluorescence *in situ* hybridization (FISH) method for localizing rice genes and DNA sequences on rice chromosomes have been described. The improved FISH method has been applied to the analysis and characterization of rice chromosomes. The principal results of this study are :

1. Improvements in the fluorescence *in situ* hybridization procedures were made to obtain clear and reproducible signals on rice chromosomes. Four steps of post-treatments were developed to reduce the noise and enhance the signal intensity. A modified thermal cycler was developed and employed for FISH to ensure reproducibility of the signal detection. An imaging method was introduced to enhance the FISH signal digitally.

2. The improved FISH method was employed for physical mapping of 45S ribosomal RNA genes in *Oryza* species. Variability in the number of 45S rDNA loci was detected using eight diploid species. The 45S rDNA loci were located on one to three chromosomes among the diploid spp. A third 45S rDNA locus was first detected in the two wild species *O. punctata* and *O. officinalis*.

3. Tandem repeat sequence (TrsA) which consists of a repeating unit of 360 bp units was used for Southern hybridization analysis with ten *Oryza* species. *Oryza* species with B, C, E and F genomes showed few hybridization signal. Thus the presence of TrsA is considered to vary among *Oryza* species.

4. Using PCR, TrsA was detected in three strains, Nipponbare, IR36 and *O. glaberrima*. TrsA sequences at *trsA1* locus were found in several rice cultivars such as IR36, Nipponbare and *O. glaberrima*. TrsA sequences at the *trsA3* locus were found in IR36 and Nipponbare but not in *O. glaberrima*.

5. The improved FISH method was applied to localize the sites of TrsA. The DNA sequence was detected adjacent to telomeric regions on the long arms of chromosomes 6 and 12 in the *japonica* variety Nipponbare. In the case of the *indica* variety IR36, TrsA sites were detected at the distal ends of the long arms of chromosomes 5, 7, 8, 9, 10 and 11. *O. glaberrima* has three sites on the distal ends of the long arms of chromosomes 5, 6 and 7.

6. Karyotype analysis of the African cultivated rice *O. glaberrima* was investigated based on the condensation pattern of individual rice chromosomes. According to the analysis, only slight differences were detected around the distal

regions of several chromosomes. The morphology of *O. glaberrima* chromosomes is almost identical to that of *O. sativa* chromosomes.

7. A multicolor FISH (McFISH) was developed and used for the simultaneous detection of 5S rDNA and 45S rDNA loci in the same nucleus or chromosome. The McFISH method was applied to physically map 5S rDNA and 45S rDNA loci. A 45S rDNA locus was mapped at the distal end of the short arm of chromosome 9. One 5S rDNA locus was also localized at the proximal region of the short arm of chromosome 11.

### ACKNOWLEDGEMENTS

I wish to express my heartfelt thanks to Professor Dr. Toshiro Kinoshita, Hokkaido University, for his invaluable guidance throughout the study and critical reading of my dissertation. I am grateful to Professor Dr. Yoshiya Shimamoto and Professor Dr. Tetsuo Mikami, Hokkaido University, for their critical reading and comments.

I wish to express my sincere gratitude to Dr. Kiichi Fukui, Hokuriku National Agricultural Experiment Station, for his invaluable guidance, discussion, continuous encouragement and critical reading of the dissertation. I am also grateful to Professor Dr. Eiichi Ohtsubo and Professor Dr. Hisako Ohtsubo, Tokyo University, for their guidance and discussion concerning molecular aspects of my research. I also thank Drs. Yoshimichi Fukuta and Masahiro Yano, Hokuriku National Agricultural Experiment Station, for their help in the Southern analysis and subculture of the *E. coli* experiments.

Special thanks must be given to Dr. Miki Nakamura (Kyushu Tokai University), Dr. Yasuko Kamisugi (Cambridge Laboratory, UK.), Mr. Shigeki Nakayama (National Institute of Agrobiological Resources) and Mr. Yuuji Ito (Morioka Branch, Fruit Tree Research Station) for their helpful advice and suggestions on plant chromosomes throughout the study. I also thank to Ms. Rieko Shishido (Hokkaido University) for her warm encouragement and helpful support. I express my gratitude to Ms. Keiko Kuroda, Ms. Keiko Kawakami and other members of the Laboratory of Breeding Methodology, Hokuriku National Agriculture Experiment Station, for their technical assistance and heartfelt encouragement.

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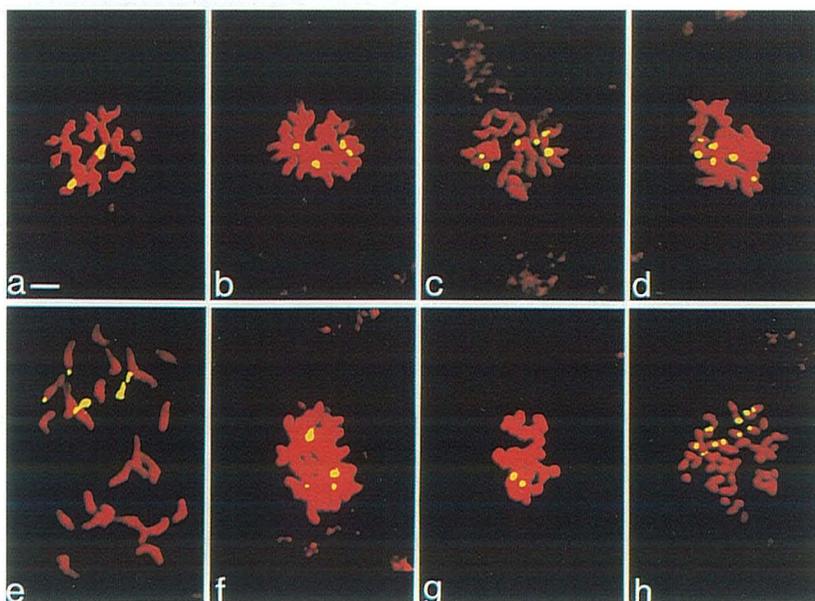
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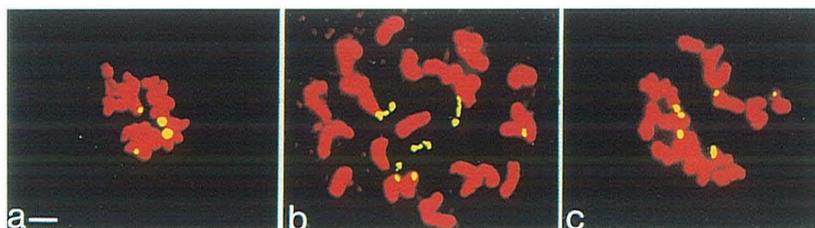
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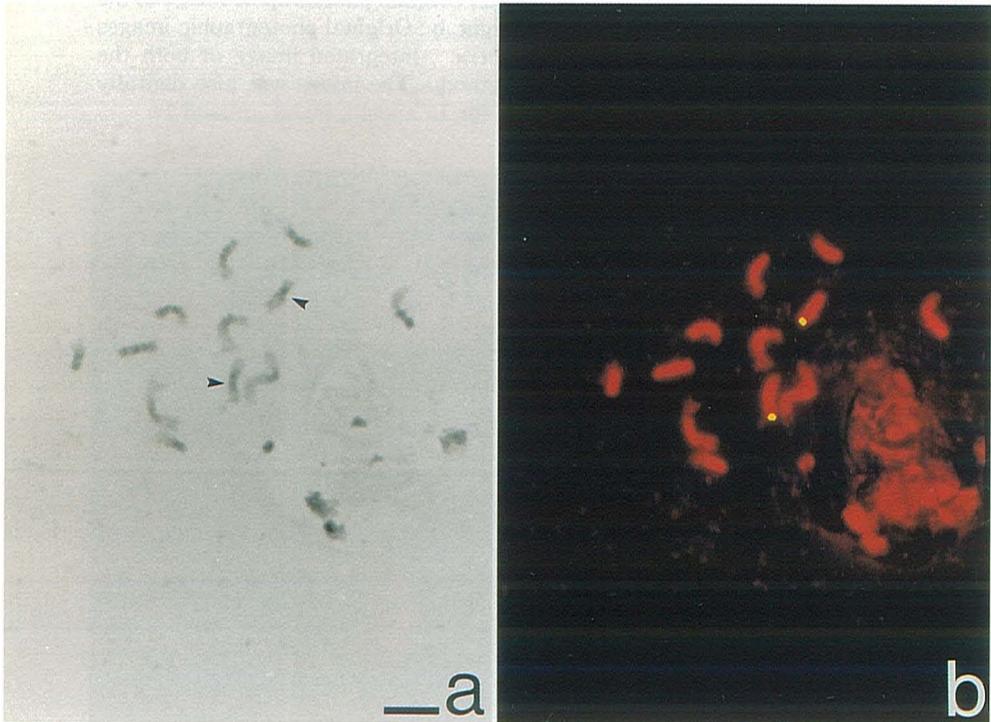
**Plate 1.** Images of *in situ* hybridization of IR36 chromosomes using the biotinylated 45S rDNA probes. a ; Original photographic image of the chromosomes in G excitation light. b ; Original photographic images of signals in B excitation light. c ; Integrated image of both the images a and b by imaging method. The image was also digitally enlarged. Bars indicate 5  $\mu\text{m}$  for 1a and 1b, and 3  $\mu\text{m}$  and 1c.



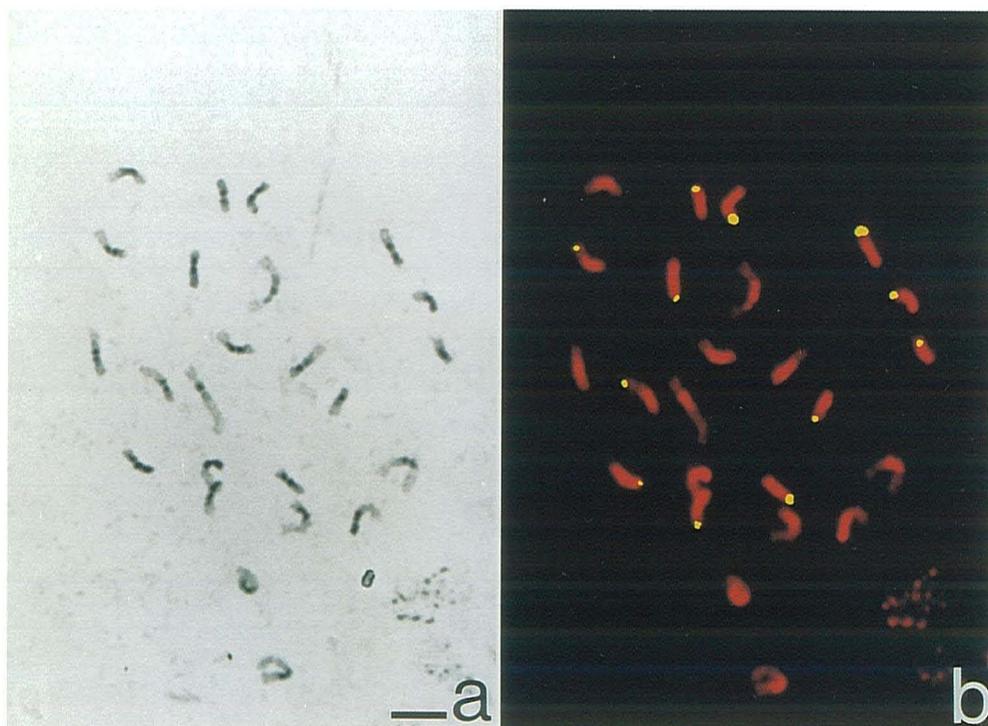
**Plate 2.** Fluorescent signals of 45S rDNA sites in seven rice species. a ; *O. sativa* ssp. *japonica*, CH79. b ; *O. sativa* ssp. *javanica*, Inakupa. c ; *O. punctata*. d ; *O. officinalis*. e ; *O. eichingeri*. f ; *O. australiensis*. g ; *O. brachyantha*. h ; *O. latifolia*. Bar indicates 3  $\mu\text{m}$ .



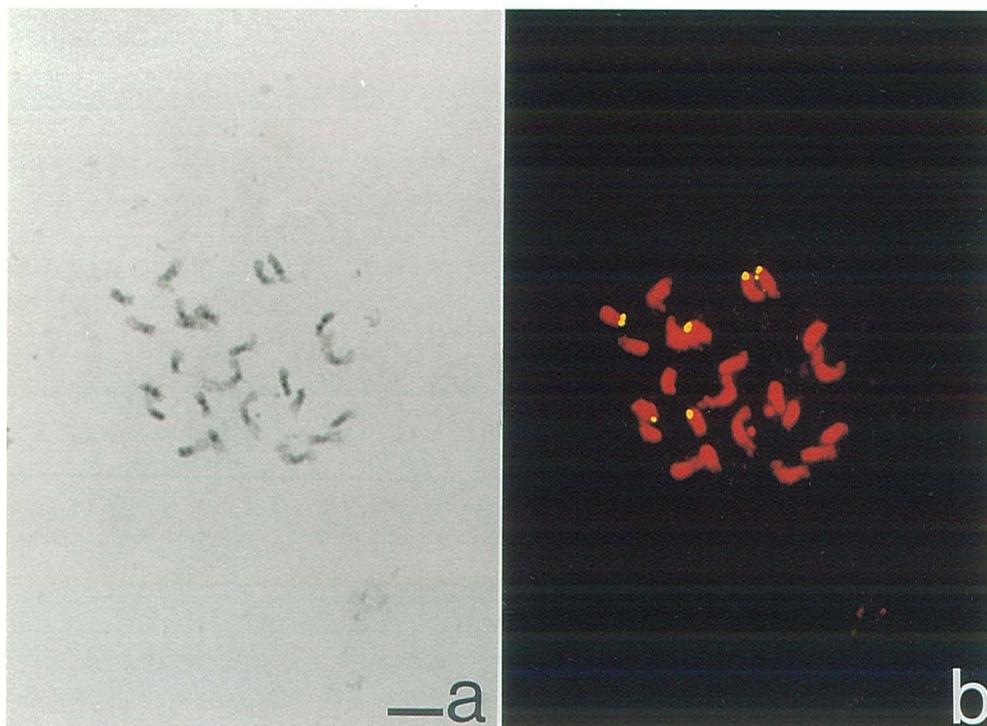
**Plate 3.** *In situ* hybridization of IR24 and its two trisomic lines. a ; IR 24. b ; Trisomic line for chromosome 9. c ; Trisomic line for chromosome 10. Bar indicates 3  $\mu\text{m}$ .



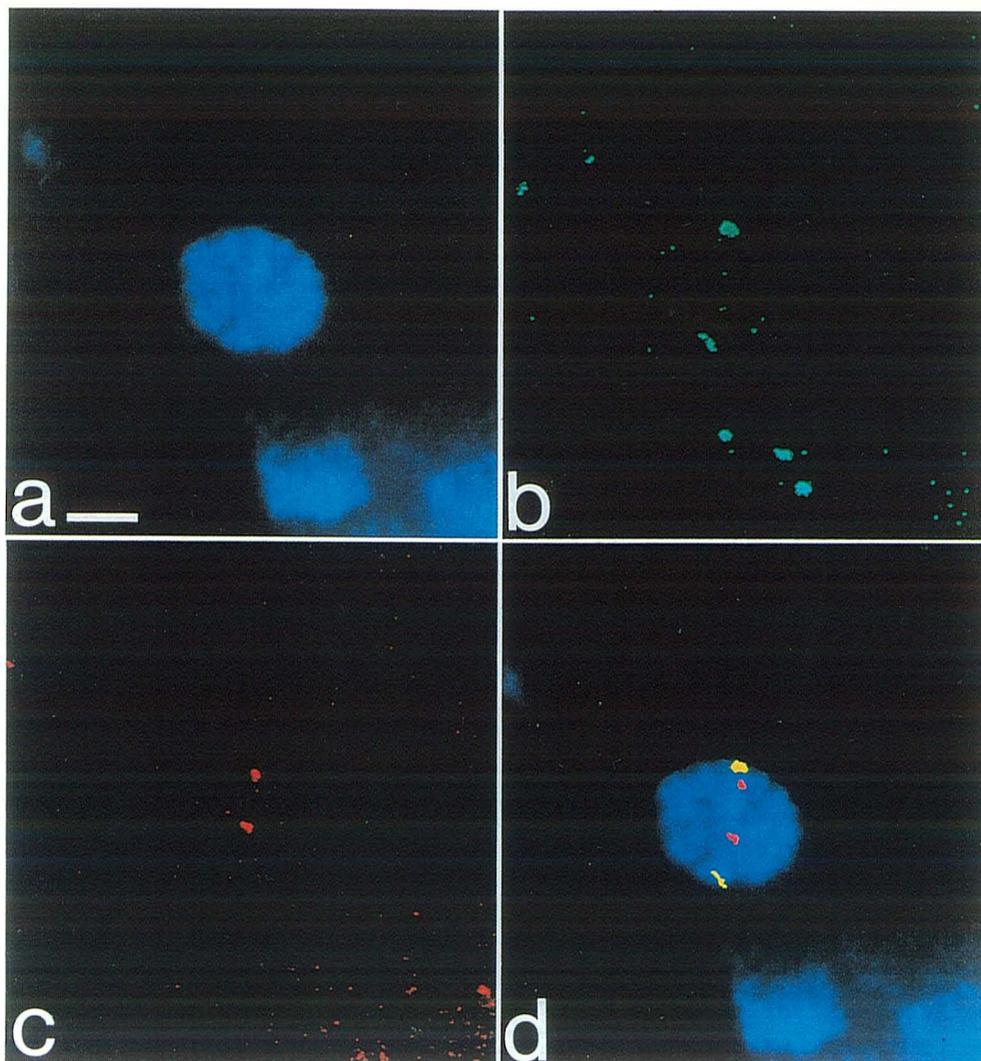
**Plate 4.** The hybridization signals of the prometaphase chromosomes of haploid Koshihikari, by FISH using TrsA as a probe. a ; Giemsa-staining chromosomes. b ; Signals of the repeated sequences. Bar indicates 5  $\mu$ m.



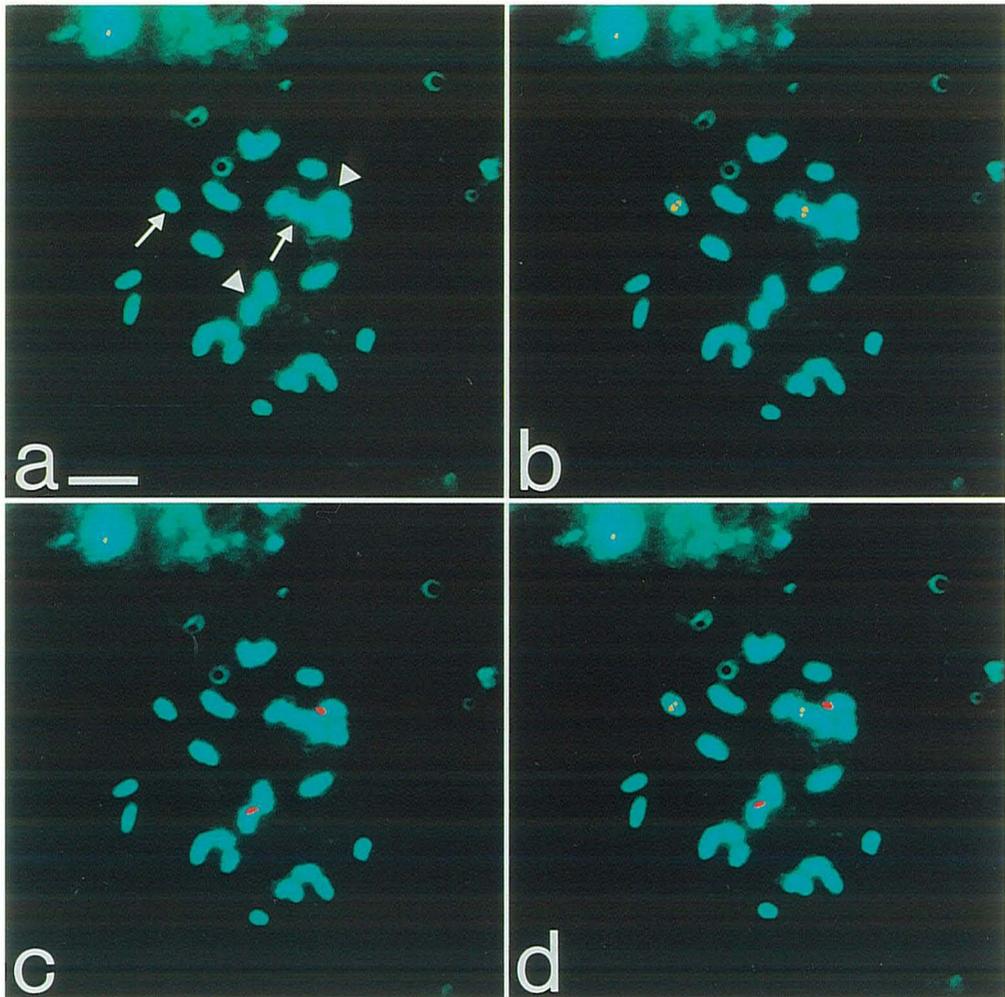
**Plate 5.** The hybridization signals of prometaphase chromosomes of diploid IR36, by FISH using TrsA as a probe. a ; Giemsa-staining chromosome. b ; Signals of the repeated sequences. Bar indicates 5  $\mu$ m.



**Plate 6.** The hybridization signals of prometaphase chromosomes of *O. glaberrima*, by FISH using TrsA as a probe. a ; Giemsa-staining chromosome. b ; Signals of the repeated sequences. Bar indicates 5  $\mu$ m.



**Plate 7.** McFISH of *O. glaberrima* nuclei with simultaneous 5S rDNA and 17S rDNA probes. a ; DAPI image of interphase chromosomes by UV excitation light. b ; FITC image of the 5S rDNA signals in B excitation light. c ; Rhodamin image of the 45S rDNA signals with G excitation light. d ; Integration of the three images by imaging methods. Scale bar indicates 5  $\mu$ m.



**Plate 8.** McFISH of *O. glaberrima* chromosomes with simultaneous 5S rDNA and 17S rDNA probes. a : DAPI image of interphase chromosomes by UV excitation light. Two pairs of chromosomes, chromosome 11 and 9 are indicated by either arrow or arrow heads, respectively. b : FITC image of 5S rDNA signals in B excitation light, c : Rhodamine image of the 17S rDNA signals in G excitation light, d : Integration of the three images by imaging methods. Scale bar indicates 5  $\mu\text{m}$ .