

Advances in rice chromosome research, 1990-95

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After providing a historical perspective on rice chromosome research, this paper reviews and summarizes advances made during the 1990-95 period. The most significant research accomplishments during the last 5 yr are the complete and objective identification of all the somatic rice chromosomes (80 yr after the first accurate report of the number of rice chromosomes) and the development of a quantitative rice chromosome map. Also, in 1991, the long discussion on unifying the various numbering systems for the rice chromosomes ended with the implementation of a new numbering system. New technologies recently developed include 1) imaging methods that make it possible to identify rice chromosomes automatically using personal computers, 2) fluorescence in situ hybridization, 3) genomic in situ hybridization for differential painting of the rice genomes, and 4) the laser microdissection method used in dissecting specific regions of individual rice chromosomes.

Historical perspective

Chromosome analysis in rice

Kuwada (1910) first reported the chromosome number of cultivated rice, $2n=24$, using both mitotic and meiotic cells. Many cytological studies followed to elucidate the characteristics of the rice chromosomes. For example, Rau (1929) classified rice chromosomes into three types and Nandi (1936) reported that chromosome size ranged from 0.7 to 2.8 μm at mitotic metaphase.

Hu (1964) identified seven or eight different rice chromosome types at mitotic metaphase and karyotypic similarity between *Oryza sativa* and *O. glaberrima*, which later was unequivocally proven by imaging methods (Ohmido and Fukui 1995). The effectiveness of the prometaphase stage in identifying rice chromosomes was also

demonstrated by Hu (1964). Kurata and Omura (1978) and Wu et al (1985) reported the respective karyotypes of japonica and indica rices. Also, Kurata and Omura (1978) used an elegant enzymatic maceration and flame-drying method for preparation of chromosomes, which gives an excellent spread.

Shastry et al (1960) examined pachytene chromosomes, which are suitable for detailed observation of rice chromosomes. Nishimura (1961) developed 12 reciprocal translocation lines in a japonica variety, Kinmaze. Khush et al (1984) and Iwata and Omura (1984) developed 12 primary trisomic series using indica and japonica rices, respectively. In 1991, a new chromosome numbering system was proposed to integrate the somatic karyotype, pachytene karyotype, Nishimura's numbering system, trisomics,

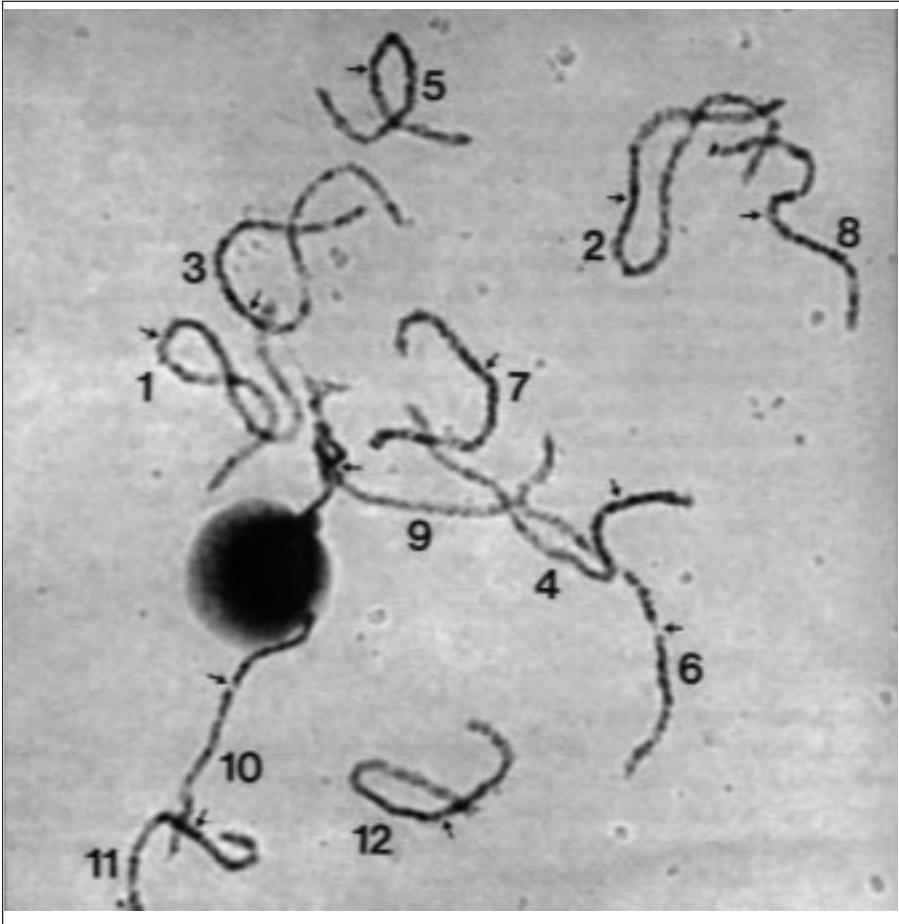


Fig. 1. Pachytene chromosomes of an indica (IR36) haploid plant. Arrows and numbers show centromeres and "new" chromosome numbers, respectively. Chromosomes 9 and 10 are attached to a nucleolus, clearly indicating that the two chromosomes have the nucleolar organizing regions (Khush and Kinoshita 1991).

and linkage groups based on the length of pachytene chromosomes (Khush and Kinoshita 1991). Figure 1 shows the spread of the pachytene chromosomes. Dark proximal chromomeres and faint distal chromomeres are both clear in all the chromosomes.

Genome analysis in rice

Morinaga (1937, 1939) first identified different genomes in the rice species and proposed five different genomes from A to E based on the results obtained. The differently proposed genome formulas of rice species were integrated by a committee at the Symposium for Rice Genetics and Cytogenetics in 1963. To date, six different genomes from A to F and corresponding diploid species except the D genome are identified in the genus *Oryza* (Vaughan 1989). The D genome is recognized only in combination with the C genome in amphidiploid CCDD species.

Identification of rice chromosomes and a quantitative chromosome map

Identification of rice chromosomes

Banding methods are often useful to identify plant chromosomes. No banding method has so far been successful in rice chromosomes in spite of many trials. Although there are some preliminary reports on G-bands in rice, they are not stable and reproducible for identifying rice chromosomes and for developing a chromosome map.

Small plant chromosomes, such as those in rice, often show uneven condensation at mitotic prometaphase as shown in Figure 2 (Hu 1964, Kurata and Omura 1978, Fukui 1986a, 1989). Thus, the condensation pattern (CP) appearing at prometaphase was only a morphological landmark to divide the rice chromosomes into subregions (Fukui and Mukai 1988, Fukui 1989). Characteristics of each rice chromosome with uneven condensation have quantitatively been analyzed by using image analysis methods (Fukui 1985, 1986b, 1988; Iijima and Fukui 1991).

Figure 3 shows the steps in conducting an image analysis of rice chromosomes at prometaphase. First, a photographic or analog image of the part of a rice prometaphase chromosomal spread was digitally captured (Fig. 3a) and the contrast of the image was digitally enhanced (Fig. 3b). After determining the chromosomal regions by binarization or generation of a black and white image (Fig. 3c), the contour line of each chromosome was extracted and superimposed onto the chromosome image to check whether the extraction of the chromosomal region was adequate (Fig. 3d). Then the chromosomal images were pseudo-colored to make the minute differences of the chromatin condensation distinct (Fig. 3e). The CP or density profile along a midrib line of the chromatid was measured by using the enlarged image (Fig. 3f). By observing the condensation patterns of the chromosomes, all the rice chromosomes could be identified.

Characterization of rice chromosomes

Figure 4 shows the characteristics of 12 japonica chromosomes demonstrated by a Giemsa-stained image (upper panel), a pseudo-color image (middle panel), and a pseudo-three-dimensional image (lower panel). The average condensation pattern, based on 30 haploid prometaphase spreads, is represented graphically (Fukui and Iijima 1991). Pseudo-color and pseudo-three dimensional representation dramatically improve the ability of identifying the fine structures of each rice chromosome.

Heavy condensation of the short arm of chromosome 6(12) and the long arm of chromosome 10(7) is clearly demonstrated both by pseudo-colors and their differential coloring steps. The numbers in parentheses are the new chromosome numbers decided upon in 1991. Chromosomes 1(1), 2(3), and 3(2) are large chromosomes with long dispersed ends on both arms and lightly condensed proximal regions. Chromosome 11(9) is a subtelo-centric, satellite chromosome with tandem-repeated 45S ribosomal DNAs (nucleolar organizing region) at the end of the short arm in the case of japonica. The number of rDNA sites is different among different rice species. Chromosomes 4(4) and 12(10) are of the submetacentric type without visually dispersed regions at the end of the short arm. There are the other satellite chromosomes in *O. sativa*, indica (chromosomes 11[9], 12), and *O. officinalis* (chromosomes 4, 9[11], 11). Chromosome 9 is the only chromosome with the tertiary constriction at the long arm. Chromosomes 5(6) and 8(5) are middle-sized metacentric types. Chromosome 7(8) is also a middle-sized metacentric type with a longer long arm. Numerical data and detailed characteristics of each rice chromosome were given without (Iijima et al 1991) and with the appearing frequencies (Fukui and Iijima 1992).

Although the characteristics of the rice chromosomes are based on those in japonica rice, the basic features of each chromosome are similar in the other rice species as suggested by Hu (1964). Major differences in chromosome morphology among the species are found in the condensation at the subterminal regions, the number of satellites, and the total size of the chromosomes.

Discrimination chart for rice chromosomes

Iijima et al (1991) developed a method for identifying rice chromosomes based on a flow chart that consists of 11 discriminants, which classify specific chromosome groups. All rice chromosomes can be identified and numbered by comparing the categories given by the discriminants, one after another. The chromosomal spread is worth analyzing if chromosomes 4, 11, and 12 are distinguishable by visual inspection and if chromosomes 1, 2, and 3 can be completely recognized. If these six chromosomes can be identified using discriminants 1 through 6 in order, then there is a great possibility of identifying all 12 chromosomes within the particular spread. Note that the steps for discriminant no. 9 have been updated from those originally provided by Iijima et al (1991) as follows: "Discard the two submetacentric chromosomes. The remaining two chromosomes are either chromosome 5(6) or 8(5)."

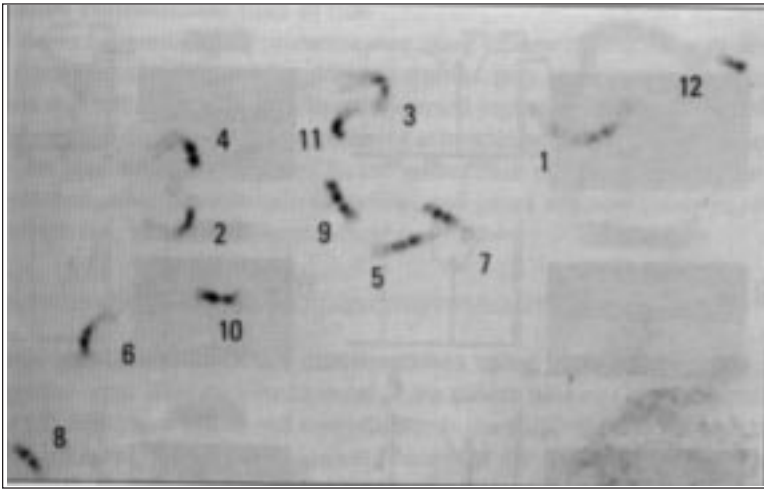


Fig. 2. Typical somatic chromosomes of a japonica (Koshihikari) haploid plant. The chromosome sample was prepared using the EMA method (Fukui and Iijima 1991). The bar in the lower right indicates 5 μ m. (Prof. Hiroo Niizeki supplied the haploid plants.)

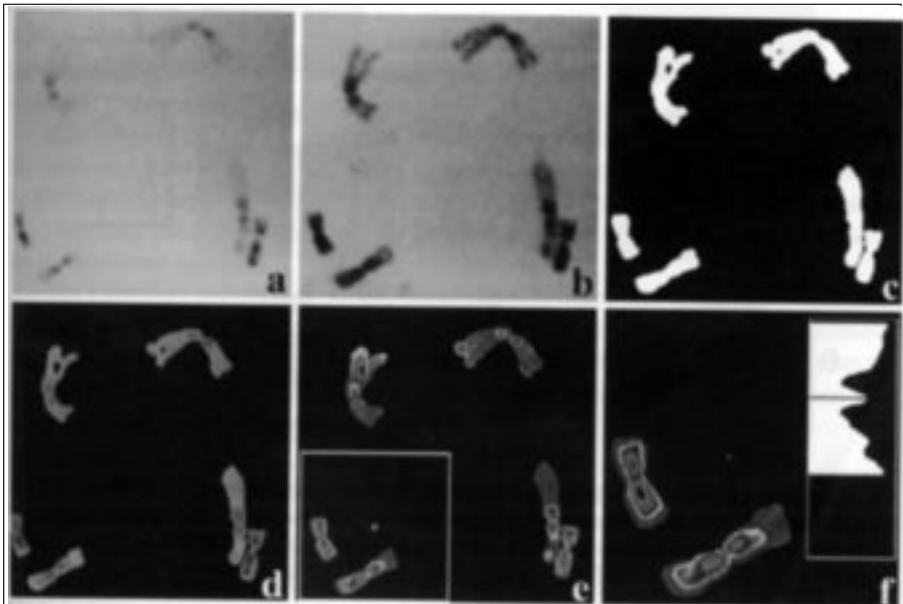


Fig. 3. Six steps in conducting an image analysis of rice chromosomes at prometaphase. Good rice chromosomal spread was microphotographed and a part of the photograph was captured by a high-resolution TV camera and subjected to digital image analysis. (See text for more detailed discussion.)

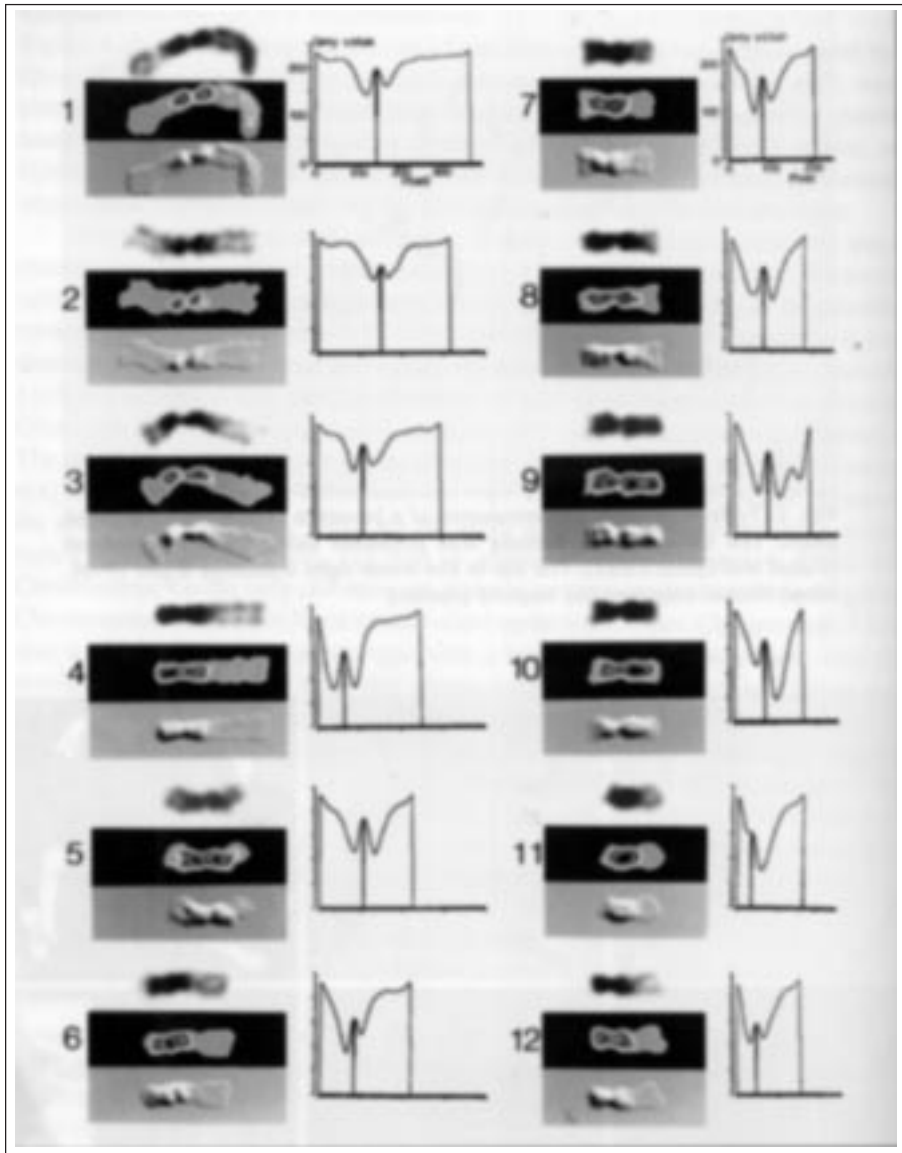


Fig. 4. Characteristics of 12 japonica chromosomes demonstrated by a Giemsa-stained image (upper panel), a pseudo-color image (middle panel), and a pseudo-three-dimensional image (lower panel). The average condensation pattern is represented graphically on the right (Fukui and Iijima 1991). (See text for more detailed discussion.)

Quantitative chromosome map of rice

Figure 5 shows a quantitative chromosome map using the new chromosome numbering system. Black bars show the most condensed regions, gray bars represent moderately condensed regions, and white bars depict dispersed regions. Visual borders between the condensed and dispersed regions appearing at prometaphase correspond to borders between the gray and white regions. As the quantitative rice chromosome map has been developed, several nucleotide sequences and genes are now being physically located on the map by *in situ* hybridization.

New technologies in rice chromosome research

Automatic identification of rice chromosomes using imaging methods

Two questions arose after the development of the quantitative rice chromosome map based on CP. First, how stable and reproducible is the map? Second, to what extent does the map represent the morphological features of the chromosomes? To answer these questions, the chromosome number visually determined under the microscope and the chromosome number automatically determined by a computer using CP numerical data were compared. All morphological information on the rice chromosomes appearing under a microscope were taken into account by experienced researchers to determine the chromosome number with the aid of image manipulation of contrast enhancement, enlargement, and pseudo-coloration as shown in Figure 4. The same chromosomal spreads were utilized for automatic identification of rice chromosomal number using the CP numerical data, which were analyzed by three computerized statistical methods—discrimination chart, linear discrimination, and minimum distance classifier.

Kamisugi et al (1993) reported results on automatic identification of rice chromosomes based on CP data. They extracted 31 image parameters from each of the CPs, such as the position of a condensation region, depth of a condensation region, etc. Then they used statistical methods mentioned above to determine automatically the chromosome numbers.

The minimum distance classifier, discrimination chart, and linear discrimination methods correctly identified 92.2, 91.1, and 84.4% of the rice chromosomes, respectively. This clearly demonstrates that CPs contain sufficient information to determine the chromosome number and are stable parameters that represent the morphology of rice chromosomes at prometaphase.

In situ hybridization and physical mapping of rice genes

In situ hybridization of rice was first accomplished by using tritiated *rbcS* probes (Wu et al 1986) and ¹²⁵iodine-labeled ribosomal DNA (Fukui et al 1987). Two signals at the end of the short arm of chromosome 11 (new numbering system used throughout the rest of this paper) were clearly detected in the latter case. However, a main obstacle in using *in situ* hybridization was the size of rice chromosomes, which were too small to localize the radioactive probes on the defined chromosomal regions. So, *in situ*

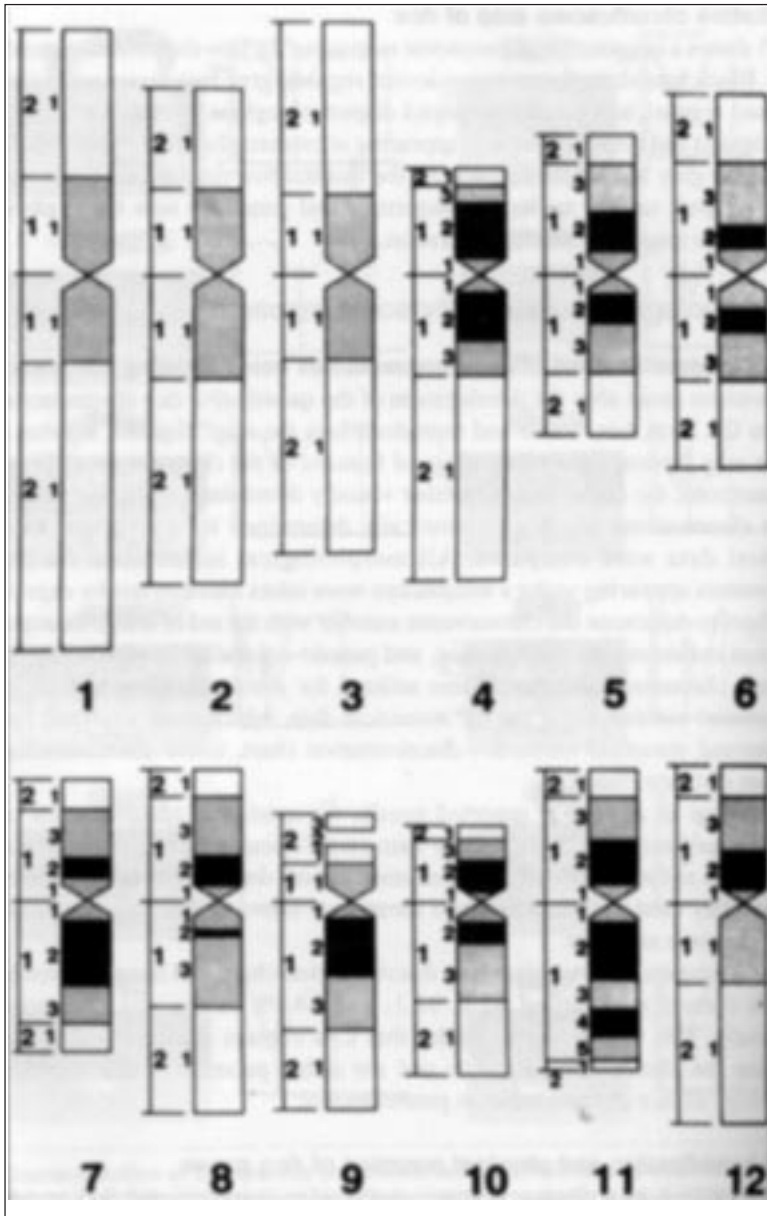


Fig. 5. This quantitative somatic rice chromosome map, based on the condensation pattern (Fukui and Iijima 1991), allows researchers to physically locate the chromosomal addresses (Fukui and Mukai 1988). Black bars show the most condensed regions; gray bars represent moderately condensed regions; and white bars depict dispersed regions.

hybridization did not become truly practical until a biotin labeling method was introduced.

In situ hybridization with biotinylated probes was used to detect rDNA loci both in japonicas (Fukui 1990) and indicas (Islam-Faridi et al 1990). The number of rDNA loci revealed was consistent with the number of the satellite chromosomes. Shortly after this, researchers detected a few copy genes for storage protein and restriction fragment length polymorphism (RFLP) markers by using either radioactive or biotinylated probes (Suzuki et al 1991, Gustafson and Dillé 1992).

Introduction of the fluorescent in situ hybridization (FISH) technique improved detection efficiency and sensitivity. Fukui et al (1994) reported the variability of 45S rDNA sites among rice species. They detected one rDNA locus in *O. sativa* (japonica), *O. rufipogon*, and *O. brachyantha*; two loci in *O. sativa* (indica [Fig. 6] and javanica), *O. eichingeri*, *O. rufipogon*, and *O. australiensis*; and three loci in *O. officinalis*. It seems that there is no species-dependent tendency, but there is a geographical variation. It is interesting that the same variation in rDNA loci detected in cultivated rice also exists in the ancestral species, *O. rufipogon*.

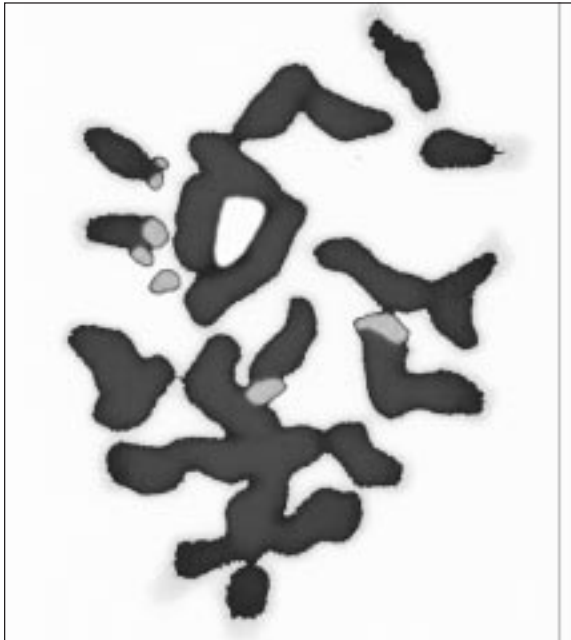


Fig. 6. Fluorescent in situ hybridization in IR36 clearly shows four fluorescein-isothiocyanate fluorescent signals on propidium iodide counterstained rice chromosomes. This photograph is the result of an improved FISH methodology designed especially for working with rice chromosomes (Fukui et al 1994). (See the cover of this volume for a color rendition.)

Another ribosomal DNA species of 5S rDNA has also physically been mapped by FISH on chromosome 9 of indica rice (Song and Gustafson 1993) and on chromosome 11 of japonica rice (Kamisugi et al 1994). The 5S rDNA locus lies at the position 4% away from the centromere toward the distal end on the short arm in japonica rice. Further research on localization of 5S rDNA on several rice species using FISH revealed that rice has only one such locus and it is located on chromosome 11 (Shishido et al 1994). Ohmido and Fukui (1995) developed multicolor FISH (McFISH) using the two probes of 45S and 5S rDNAs simultaneously in African cultivated *O. glaberrima* and detected the identical localization of the rDNAs on the chromosomes as in japonica rice.

Several repeated sequences of rice such as TrsA, TrsB, TrsC, and RIRE1 (Ohtsubo et al 1991; H. Ohtsubo, University of Tokyo, Japan, 1994, unpubl. data) have successfully been mapped on the rice chromosomes (Ohmido et al 1993). In most cases, TrsA and TrsC were localized at the subtelomeric regions of the chromosomes. There is variability in the number of the TrsA sites even within *O. sativa*. Japonicas have the fewest number of TrsA sites, two per haploid genome, whereas indicas have six sites, which are all different from the japonica sites. TrsA's locations at the subtelomeric regions suggest that it has a certain biological role.

Jiang et al (1995) reported mapping bacterial artificial chromosome (BAC) clones on rice chromosomes using FISH. The successful mapping of BACs, yeast artificial chromosomes (YACs), cosmid clones, and RFLP markers on rice chromosomes have also been obtained (N. Ohmido and K. Fukui, Hokuriku National Agricultural Experiment Station, unpubl. data). Therefore, it is anticipated that reproducible mapping of single-copy genes on rice chromosomes will be accomplished in the very near future.

Genomic in situ hybridization (GISH)

Genomic in situ hybridization (GISH)—where total genomic DNA is used as the probe—has been used for phylogenetic studies and identification of alien chromosome(s) from different genomes. Since the probe is a mixture of unique and repetitive sequences, the signal is uniformly obtained throughout the chromosome(s).

Shihido et al (1993) extracted total genomic DNA from *O. officinalis*, a diploid C genome species, and used GISH as the probe to paint the chromosomes originating from the C genome. Two amphidiploid species, *O. minuta* (BBCC) and *O. latifolia* (CCDD), were used in preparing the chromosome samples. Twenty-four chromosomes, derived from the ancestral diploid C genome species, were clearly painted in both *O. minuta* and *O. latifolia*. This work identified chromosomes originating from a D genome species for the first time. The differences in the strength of the fluorescent signals between the B and C genome chromosomes being clearer than those between the C and D genome chromosomes indicate that the C and D genomes are more closely related to each other than are the B and C genomes.

Optical manipulation of rice chromosomes

Recent advances in laser optics have made it possible to produce pores on protoplasts (optical poration), to “pinch” microscopic plant materials (optical tweezers), and even to dissect plant chromosomes (laser knife; Fukui et al 1995). Laser microdissection seems to be one of the most useful new technologies in chromosome research.

Figures 7a-d show the representative steps of laser dissection of rice chromosomes (Fukui et al 1992). Figure 7a shows the rice chromosomal spread prepared by the enzymatic maceration/air-drying (EMA) method (Fukui and Iijima 1992). To dissect two chromosomal regions of chromosome 4, scattered cytoplasmic debris was first eliminated and nontarget chromosomes in the peripheral regions were removed by laser ablation (Fig. 7b). Then, the remaining chromosomes within the spread, except for chromosome 4, were removed by laser ablation (Fig. 7c). A fine laser beam was then used to eliminate the condensed region to clarify the dispersed tail region of chromosome 4. For another chromosome 4, the dispersed region was laser-ablated to obtain the condensed region of the chromosome (Fig. 7d).

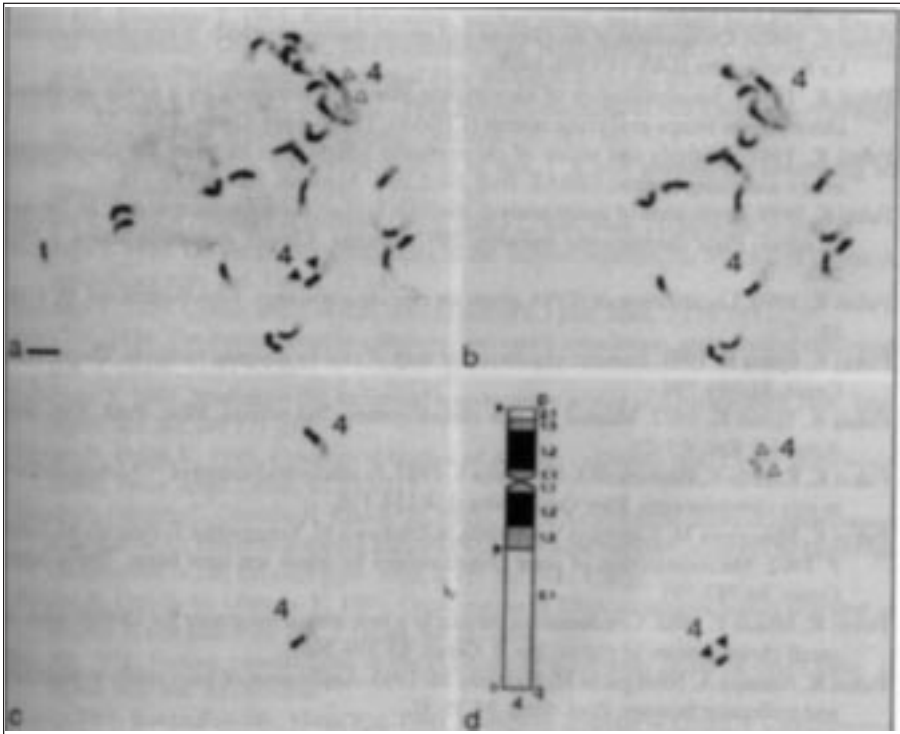


Fig. 7. Chronological steps for laser dissection of rice chromosome 4 using ACAS 470. The bar in 7a indicates 5 μm . The solid and open triangles in 7a and d indicate the chromosomal regions subjected to laser dissection (Fukui et al 1992). In 7d, p and q indicate the short and long arms, respectively. (See text for more detailed discussion.)

The rice chromosomal spread subjected to direct cloning of nucleotide sequences from a certain chromosomal fragment is prepared on the polyester membrane. Then, an octagon disk, on which the chromosome fragment is located at the center, is dissected by the much stronger laser beam and the disk is recovered with fine forceps. The disk itself is used as the DNA template for polymerase chain reaction (PCR) amplification. DNA amplified by PCR is simultaneously labeled with biotin or degoxigenin for further use of the labeled DNA in in situ hybridization.

The new techniques just described—identifying the rice chromosome and specifying the region using the imaging methods, dissecting the region of interest with a fine laser beam, direct cloning and direct labeling of the DNA, and confirming the origin of the amplified DNA using FISH—are now available to researchers. The next step is to integrate these procedures into one logical, complete system.

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Notes

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