Molecular Cytological Diversity in Cultivated Rice *Oryza sativa* Subspecies *japonica* and *indica*

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In cultivated rice *Oryza sativa*, although physiological and molecular biological studies have demonstrated the existence of a high intra-species diversity, there are few reports related to the molecular cytological diversity. To examine the molecular cytological diversity in *O. sativa*, a tandem repeat-sequence Os48 was visualized using fluorescence *in situ* hybridization (FISH) in various rice varieties. Diversity was reflected by differences in the number of FISH signals. The number of loci detected was almost the same among the *O. sativa* subspecies *japonica* varieties, but differed significantly among the *O. sativa* subspecies *indica* varieties. The difference in the number of Os48 loci reflected differences in the chromosomal structure. In *O. sativa*, repeat sequences of 45S rDNA were also mapped to distal region(s) of the chromosome(s). Two-colored FISH of rDNA and Os48 revealed a common chromosomal structure within *japonica* varieties and *indica* varieties, but a distinctly different structure between *japonica* and *indica* varieties. The present study indicated that there was less cytological diversity among *japonica* varieties than among *indica* varieties. FISH results also allowed considerations of the domestication of cultivated rice *O. sativa*.

Key Words: domestication, fluorescence *in situ* hybridization (FISH), *indica*, *japonica*, *Oryza sativa*, Os48, rDNA.

Introduction

Cultivated rice, *Oryza sativa* L., is one of the most important crops in the world. The classification and genetic diversity of *O. sativa* are important for both rice breeders and geneticists. *O. sativa* is classified into two subspecies, *japonica* and *indica*, with a third group *javanica* classified as a tropical component of the subspecies *japonica* (Oka 1958). The genetic diversity of *japonica* and *indica* has been investigated based on morpho-physiological traits (Oka 1958), isozymes (Second 1982, 1985, Glaszmann 1987), and nucleic and cytoplasmic molecular markers (e.g., Ishii et al. 1988, Wang and Tanksley 1989, Zhang et al. 1992, Suh et al. 1997, Bautista et al. 2001, Sun et al. 2001, Cheng et al. 2003), and in these studies it was reported that genetic diversity was higher in *indica* than in *japonica*.

Cytological diversity in the genus *Oryza* has been investigated mainly based on the chromosome number (polyploidy) and structure (Vaughan 1994). However, the chromosome number in *O. sativa* does not vary (2n=24, diploid) except in mutants. Cytological diversity in the genus *Oryza* has also been investigated based on the chromosomal structure, visualized by fluorescence *in situ* hybridization (FISH) of repeat DNA sequences and ribosomal RNA genes (rDNA) (Fukui et al. 1994, Uozu et al. 1997, Shishido et al. 2000).

Almost all the studies on molecular cytological diversity in *O. sativa* were performed by comparing two representative varieties, one from the subspecies *japonica* and the other from the subspecies *indica* (Ohmido and Fukui 1997, Uozu et al. 1997, Shishido et al. 2000, Cheng et al. 2001). In a previous FISH study of *O. sativa*, the existence of one rDNA locus was reported in almost all the *japonica* varieties and of two rDNA loci in a glutinous *japonica* variety from South China, *javanica* (tropical *japonica*) varieties, and *indica* varieties (Fukui et al. 1994). FISH of rDNA did not reveal a molecular cytological diversity among the *indica* varieties.

Os48 (Rc48) is a representative tandem repeat-sequence in *O. sativa* (Wu and Wu 1987). This 352-bp length sequence has only been reported in the *Oryza* AA genome (Wu et al. 1991). There is also another tandem repeat-sequence in the AA genome, TrsA, which shows a 90% homology to Os48 (Ohhtsubo et al. 1991). FISH of Os48 enabled to detect eight loci on the meiotic chromosomes of the *indica* variety Zhongxian 3037 and two loci in the *japonica* variety Wuyujing 8 (Cheng et al. 2001). FISH of TrsA revealed six loci in the *indica* varieties IR8 and IR36 and two in the *japonica* variety Nipponbare (Ohmido and Fukui 1997, Ohmido et al. 2000). Any difference in the number of Os48 loci among *indica* varieties should reflect a difference in the chromosomal structure at these loci. Thus molecular cytological diversity can be investigated based on the difference in the number of Os48 loci. In the present study, the molecular cytological diversity in *O. sativa* was examined using FISH of Os48 and 45S rDNA.

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Materials and Methods

Plant materials

Sixteen varieties of *O. sativa*, including temperate and tropical varieties of *japonica* and boro and aman varieties of *indica*, were studied (Table 1).

Isolation of *Os48* and cytological procedures

*Os48* was isolated by polymerase chain reaction (PCR) amplification from genomic DNA of the *indica* variety Kasalath. Genomic DNA was isolated from young leaves after germination with the DNeasy Plant Mini System (QIAGEN, Germany), according to the manufacturer’s instructions. A primer set, Os48-F: GAATT CTGTG CAAAT TACCCG AAGGC, was designed based on *Os48* sequence data (Wu and Wu 1987). PCR amplification was performed using Ex Taq DNA polymerase (TaKaRa, Japan) with 25 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 5 min, after an initial denaturation of 92°C for 10 min. The amplified *Os48* was cloned into the pGEM-T easy vector system (QIAGEN, Germany), according to the manufacturer’s instructions. This clone was sequenced and showed 91% homology to *Os48* (Genbank accession: X12438; data not shown).

Chromosomal preparations were produced as previously described (Fukui 1996). FISH procedures were performed as previously described (Nakayama et al. 2004). *Os48* was labeled using a random primed labeling kit (Nippongene, Carlsbad, CA) and isolated using an RPM Turbo Kit (Qbiogene, Carlsbad, CA), according to the manufacturer’s instructions. This clone was sequenced and showed 91% homology to *Os48* (Genbank accession: X12438; data not shown).

Table 1. List of the *O. sativa* varieties used in the study

<table>
<thead>
<tr>
<th>Column (1)</th>
<th>Subspecies</th>
<th>Variety (Acc #)</th>
<th>Source (2)</th>
<th><em>Os48</em> loci No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>japonica</strong></td>
<td>lowland</td>
<td>Fujihikari (JP10272)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>lowland</td>
<td>Nipponbare</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>lowland</td>
<td>Sasanishiki</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>lowland</td>
<td>TaiChung 65</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>lowland</td>
<td>Akayakan</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>upland</td>
<td>Kahei</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>tropical</td>
<td>Rikuto Norin 24</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>tropical</td>
<td>Sensho</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>H</td>
<td>tropical</td>
<td>Garumbalay (T0219)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>tropical</td>
<td>Padi ase banda (T0647)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>J</td>
<td>tropical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>indica</strong></td>
<td>boro</td>
<td>Chinsurah Boro II (JP12878)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>K</td>
<td>boro</td>
<td>Habigangan Aman III</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>L</td>
<td>modern</td>
<td>IR24</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>M</td>
<td>modern</td>
<td>IR36</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>N</td>
<td>modern</td>
<td>Kasalath</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>O</td>
<td>modern</td>
<td>Peh-kuh (T0108)</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

1) Plants were grown in a domed green house at NIAS.
2) Letters correspond to component parts of Figure 1.
3) 1: National Institute of Agrobiological Sciences (NIAS); 2: National Institute of Genetics (NIG).

Comparison of signal amplification methods for FISH of *Os48*

Two methods of signal amplification, TSA and ABC, were compared to determine the most suitable technique for FISH of *Os48* on the chromosomes of the *japonica* variety Nipponbare. TSA revealed six signals in the distal regions (Fig. 1B); four major signals and two faint signals. ABC revealed only four signals in the distal regions as reported in the *japonica* varieties (data not shown) (Ohmido and Fukui 1997, Ohmido et al. 2000, Cheng et al. 2001). Three loci were detected in 57.9% of the FISH plates amplified using the TSA method, and two loci were detected in all the FISH plates amplified by the ABC method. Comparison with rice genome sequence data in the riceGAAS database (Sakata et al. 2002) revealed the presence of *Os48* (X12438) loci on five chromosomes; i.e., chromosomes 5, 6, 8, 9 and 10 (based on the available data as of January 31, 2005). The homologous region was longer than 80 kb on chromosomes 5 and 6. On chromosome 10, it was approximately 30 kb and on chromosomes 8 and 9, it was shorter than 13 kb. FISH and sequence analyses indicated that the ABC method enabled to visualize two loci on chromosomes 5 and 6, while the TSA method enabled to visualize three loci, which should consist of two major loci on chromosomes 5 and 6 and one minor locus on chromosome 10. These results supported the findings of a previous study in which FISH of *Os48* enabled to detect two loci on the meiosis chromosomes 5 and 6 in the *japonica* variety (Cheng et al. 2001). Efficient TSA amplification resulted in the visualization of the minor locus on chromosome 10. In the present study, TSA was selected for 17S rDNA, a part of 45S rDNA, was labeled by PCR amplification with 50% of dT replaced with digoxigenin (DIG)-11-dUTP (Roche Diagnostics), as previously described (Nakayama et al. 2001). After post-hybridization washes, the biotin-labeled probe was detected with Cy3-Streptavidin (Amersham Biosciences, Piscataway, NJ) using the tyramide signal amplification (TSA) method, which amplifies signals via the deposition of biotin-conjugated tyramide onto the chromosome by catalysis of horseradish peroxidase (Perkin Elmer, Wellesley, MA), or one cycle of avidin-biotin-conjugated (ABC) amplification, which amplifies signals with Cy3-Streptavidin and biotinylated anti-avidin (Vector Laboratories, Burlingame, CA). The DIG-labeled probe was detected with Anti-Digoxigenin-Fluorescein (Roche Diagnostics). The chromosomes were counter-stained with 1.0 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories) and observed with a Zeiss Axioplan II using Cascade blue, #17 and #20 filter sets, for DAPI, fluorescein and Cy3, respectively. Fluorescent images were captured using a cooled CCD camera (PentaMax, Photometrics, Tucson, AZ) and analyzed using IPlab (Scanalitics, Fairfax, VA) and Adobe Photoshop (Adobe, San Jose, CA).
Molecular cytological diversity in *Oryza sativa*

the visualization of Os48 because of the amplification efficiency, in spite of the slight diffusion of the TSA signals by activated tyramide (Macechko *et al.* 1997) and the difficulty in detailed mapping on the small chromosomes of *O. sativa*.

**Fig. 1.** FISH of Os48 on *O. sativa* chromosomes.


**Fig. 2.** Two-colored FISH of Os48 and rDNA on *O. sativa* chromosomes.

Differences in the number of visualized Os48 loci among rice varieties

FISH results for 16 varieties of *O. sativa* are shown in Figure 1 and summarized in Table 1.

Ten *japonica* varieties were used in the present study. All the four temperate lowland varieties showed three pairs of signals in the distal regions of three chromosome pairs (Fig. 1A–1D). The three pairs of signals included one faint pair. In the four temperate upland varieties also, all the loci were mapped to distal regions (Fig. 1E–1H). The Akayakan, Kahei and Rikuto Norin 24 varieties showed three pairs of signals on three chromosome pairs (Fig. 1E–1G), whereas Senso showed two pairs of signals on two chromosome pairs (Fig. 1H). In addition, the intensity of the three pairs of signals differed among the three varieties. The signals in Kahei included one faint pair as observed in the lowland varieties (Fig. 1F), while all the three pairs of signals in Akayakan and Rikuto Norin 24 were strong (Fig. 1E and 1G). The difference in the signal intensity at the third locus among the three varieties should reflect a difference in the copy number at the locus. This difference in copy number at the third locus might explain the results for Senso; that is, a third Os48 locus in Senso was present but the copy numbers at the third locus were too few to be visualized. In two tropical varieties, three loci were observed in the distal regions on three chromosome pairs (Fig. 1I and 1J). Signal intensity at the third locus differed between these two varieties. There were faint signals at the third locus of Garumbalay (Fig. 1I), whereas the signals at the third locus of Padi ase banda (Fig. 1J) were strong. Therefore, among the 10 *japonica* varieties, the number of loci visualized by FISH was almost the same. The difference was in the signal intensity at the third locus, which should be correlated with the Os48 copy number.

The results from the six *indica* varieties were different from those of the *japonica* varieties. On *indica* chromosomes, the number of FISH loci differed among the varieties (Fig. 1K–1P). In Chinsurah Boro II, four Os48 loci were visualized in the distal regions of four chromosome pairs (Fig. 1K). FISH revealed five pairs of signals in the distal regions of four chromosome pairs in Habiganj Aman III (Fig. 1L) and five chromosome pairs in Kasalath (Fig. 1O). There was a decrease in the number of chromosomes with the Os48 locus in Habiganj Aman III, because in one chromosome pair, two loci were identified in two distal regions (arrows in Fig. 1L and Fig. 2E). IR24 and IR36 showed six pairs of signals in the distal regions of six chromosome pairs (Fig. 1M and 1N). Peh-kuh showed seven pairs of signals in the distal regions of seven chromosome pairs (Fig. 1P). These studies revealed that the number of Os48 loci visualized using FISH differed significantly among *indica* varieties. This difference reflected a difference in the chromosomal structure among *indica* varieties. On Habiganj Aman III chromosomes, further structural variation was detected with one chromosome harboring two Os48 loci.

These single-colored FISH analyses revealed two aspects relating to the molecular cytological diversity in *O. sativa*. First, the molecular cytological diversity had increased in both *japonica* and *indica*. Second, the molecular cytological diversity in *japonica* differed from that in *indica*. In *japonica*, the number of loci identified was almost the same among the varieties and molecular cytological diversity was indicated by differences in the signal intensity at the third locus. In *indica*, the number of Os48 loci differed among the varieties and one chromosome harbored two Os48 loci. In summary, the molecular cytological diversity was low among the *japonica* varieties, and high among the *indica* varieties.

Two-colored FISH of Os48 and 45S rDNA

In *O. sativa*, repeat-sequences of 45S rDNA were also mapped to the distal region(s) of the chromosome(s). Two-colored FISH of Os48 and rDNA was performed to determine the correlation between the loci and the differences in the chromosomal structure between *japonica* and *indica* varieties.

Three *japonica* varieties were used (Fig. 2A–2C). One pair of rDNA signals was mapped to chromosome 9 on Fujihikari as reported in the temperate lowland *japonica* varieties (Fig. 2A) (Fukui and Iijima 1991, Fukui et al. 1994). Akayakan and Garumbalay showed two pairs of rDNA signals (Fig. 2B and 2C). In these *japonica* varieties, the rDNA loci were mapped to chromosomes that differed from those on which Os48 was visualized.

Three *indica* varieties were also used (Fig. 2D–2F). Two pairs of rDNA signals were detected on Chinsurah Boro II and Kasalath chromosomes and these rDNA loci were mapped to chromosomes 9 and 10, as reported in *indica* varieties (Fig. 2D and 2F) (Islam-Faridi et al. 1990, Ohmido et al. 2000, Shishido et al. 2000, Cheng et al. 2001). On these chromosomes, the Os48 loci were also mapped to the other distal region. Habiganj Aman III showed one pair of rDNA signals. On this chromosome pair, Os48 was mapped to the other distal region (Fig. 2E), as in the case of the other *indica* varieties. In these *indica* varieties, the cytological localization of rDNA and Os48 on one chromosome was observed on all the chromosomes on which rDNA was located.

Two-colored FISH of rDNA and Os48 revealed that the chromosomal structure differed distinctly between *japonica* and *indica*, which was suggested by a single-colored FISH study between two representative varieties (Cheng et al. 2001). rDNA loci on the *indica* chromosomes were localized with the Os48 locus, while the rDNA on the *japonica* chromosomes was mapped independently of the visualized Os48 loci.

Discussion

In the present study, 16 varieties of *japonica* and *indica* subspecies were analyzed using single-colored FISH of Os48 to determine the degree of molecular cytological diversity in *O. sativa*. Two-colored FISH of Os48 and rDNA
directly depicted obvious differences in the chromosomal structure between japonica and indica. These studies revealed the intra-species cytological diversity and the cytological similarity in O. sativa.

Among the japonica varieties, FISH revealed a close similarity between temperate upland and tropical varieties based on the presence of strong signals at the third locus and two rDNA loci, and the molecular cytological diversity at the third locus was higher in the temperate upland varieties than in the lowland varieties. These FISH analyses supported the findings reported in previous studies in which genotypes and plastid DNA were found to be more polymorphic in upland than lowland varieties in the temperate japonica and upland varieties closely related to the tropical japonica varieties (Ishikawa et al. 1997, 2002).

In the indica varieties, FISH revealed the presence of four to seven Os48 loci among the varieties, reflecting obvious differences in the chromosomal structure. In addition, Habiganj Aman III exhibited a unique Os48 localization and a different number of rDNA loci from that of the other indica varieties. A comparison of the FISH results among indica varieties suggested that chromosomal translocation had occurred in the history of Habiganj Aman III. Interestingly, the localization of rDNA and Os48 on one chromosome was also observed in all the indica varieties examined, while the chromosomal structure differed among the indica varieties. This localization, however, was not detected in the japonica varieties, even in the temperate upland and tropical varieties with two rDNA loci.

Single-colored FISH of Os48 revealed a lower diversity in the japonica than in the indica subspecies. Two-colored FISH of rDNA and Os48 indicated that the chromosomal structure differed distinctly between japonica and indica. These FISH results provided insights into the origin of cytological diversity and domestication of O. sativa. First, distinct differences in the chromosomal structure between japonica and indica indicated the presence of differences in the chromosomal structure between their ancestors. This supports the hypothesis that indica and japonica originated from different sources; that is, the diphylectic or polyphylectic domestication of O. sativa. Second, the difference in the degree of molecular cytological diversity in O. sativa and the maintained cytological localization of rDNA and Os48 in the indica varieties led to two hypotheses. One is that the domestication of indica varieties is at least diphylectic, and the molecular cytological diversity depicted by the difference in the number of Os48 loci occurred in the ancestors of the subspecies indica. The other is that the changes in the chromosomal structure during differentiation in indica proceeded much faster than in japonica. These cytological studies indicate the presence of independent diphylectic or polyphylectic domestication of O. sativa, that had been previously suggested based on isozyme and molecular studies (e.g., Ishii et al. 1988, Cheng et al. 2003). Application of these FISH studies to ancestral wild relatives of O. sativa and other species in the AA genome of the genus Oryza may contribute to further elucidating the history of rice domestication and that of speciation in the genus Oryza.

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