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Comparative studies of the structure of chloroplast DNA from four species of *Oryza*: cloning and physical maps

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Summary. Chloroplast DNAs (ctDNAs) were prepared from the mature green leaves of three species in the genus *Oryza*, namely, *O. punctata* (genome type BB), *O. officinalis* (CC), and *O. australiensis* (EE). After digestion with restriction enzymes, ctDNAs were cloned into a lambda phage vector and overlapping clone banks of the entire chloroplast genome from each of the three *Oryza* species were obtained. *Bam*HI and *Pst*I restriction maps of the ctDNAs were constructed, and the structures of the ctDNAs from *O. sativa* and the other three *Oryza* species were compared. Two types of variation were noted: the gain or loss of restriction sites, and deletion or insertion of nucleotides. We detected two independent deletions in the *Bam*HI-3/*Pst*I-3 fragment of *O. punctata* and in the *Bam*HI-5/*Pst*I-11 fragment of *O. officinalis*, each of which was shorter than the respective fragment from *O. sativa*, and the deletions were located in spacer regions. Short direct-repeat sequences were detected at the border of both deletions, indicating that these deletions were results of intramolecular recombination mediated by these direct repeats. Further analysis on distribution of those deletions among 15 *Oryza* species revealed that the deletions found in this study represent genotype-specific variations.

Key words: Wild rice – Chloroplast DNA – Clone bank – Physical map – Deletion/insertion

Introduction

Restriction endonuclease analysis of chloroplast DNAs (ctDNAs) has been used to analyze phylogenetic relationships between closely related species or genera. Results of such analyses have revealed that ctDNAs from related taxa, even within the same genus, exhibit wide variation (Ogihara and Tsunewaki 1982; Kung et al. 1982; Palmer et al. 1983; Kishima et al. 1987). Rice species (genus *Oryza*) include seven genome types: AA, BB, BBCC, CC, CCDD, EE, and FF. Phylogenetic studies of rice species using restriction endonuclease analysis of ctDNAs have been carried out as follows. Ishii et al. (1986) isolated ctDNAs from rice species with the AA genome type and established the relationships between them by reference to differences in lengths of restriction fragments of ctDNAs. Relationships between a wider range of species of *Oryza* were analyzed by Ichikawa et al. (1986) and Dally and Second (1990). Ichikawa et al. (1986) isolated total DNAs from some *Oryza* species and detected restriction patterns by Southern hybridization with ctDNA from *O. sativa* as probes. However, they used only nine strains that represented only seven species of *Oryza*. Dally and Second (1990) isolated ctDNAs from 247 accessions in 13 *Oryza* species and detected differences by examining restriction patterns by agarose gel electrophoresis. The relationships between patterns of bands were studied by cladistic analysis and the computation of genetic distances.

Chloroplast DNA from *O. sativa* was cloned by Hirai et al. (1985) and a physical map was constructed. Subsequently, the complete sequence of the ctDNA was determined by Hiratsuka et al. (1989).

In the present study, we isolated ctDNAs from *O. punctata* (W1515), *O. officinalis* (W0002), and *O. australiensis* (W0008) and constructed overlapping clone banks of the entire chloroplast genome of each of the three species. Using these clones, we constructed physical maps of the ctDNA and compared them. In addition to the gains and losses of restriction sites, we detected deletions in ctDNAs of *O. punctata* and *O. officinalis*. The results of nucleotide sequencing around the deletions

lead us to propose that intramolecular recombinations occurred during the evolution of ctDNAs in *Oryza* species.

Materials and methods

Plant materials

Among 15 *Oryza* species (30 strains) used in this work (Table 1), *O. punctata* (W1515), *O. officinalis* (W0002), and *O. australiensis* (W0008) were used for the isolation and cloning of ctDNA. All 15 species were used for preparation of total DNAs, seeds of which were generously provided by Drs. K. Hattori and T. Tashiro (Nagoya University, Japan). Plants were grown in a greenhouse.

Preparation of chloroplast and total DNAs

Chloroplast DNAs were extracted from mature green leaves of *O. punctata, O. officinalis,* and *O. australiensis* by essentially the same method as that described by Hirai et al. (1985).

Total DNAs were extracted from 1 g of mature green leaves by the method described by Honda and Hirai (1990).

Restriction endonuclease analysis

Chloroplast DNAs were digested overnight with *Bam*HI (Takara Shuzo Co., Japan) at 37 °C. Restriction fragments were separated by electrophoresis on 0.7% agarose gels. Ethidium-bromide-stained bands were visualized with a UV transilluminator. Fragments of *Hin*dIII-digested lambda phage DNA were used as molecular-weight markers.

Cloning of ctDNAs and construction of physical maps

Cloning of ctDNA was carried out as directed by the supplier of the cloning system (Stratagene, USA). Chloroplast DNAs were partially digested with *Sau*3AI (Takara Shuzo Co.). After "a partial fill-in reaction," ctDNA fragments were ligated with "lambda FIX/*Xho*I-partial fill-in treated DNA" (Stratagene) and the packaging reaction was carried out with Gigapack Gold (Stratagene).

Recombinant phages were selected by plaque hybridization with clones of ctDNA from *O. sativa* as probes. Hybridizations were carried out using a Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim, Germany). In order to obtain further clones, ctDNA from *O. officinalis* was digested completely with *Pst*I and *Bgl*II, and fragments were cloned into appropriate sites of pUC19.

Recombinant phage and plasmid DNAs were prepared as described by Maniatis et al. (1982). Restriction sites of the inserts were determined by restriction endonuclease analysis and Southern hybridization. The physical maps of each clone were connected and physical maps of the entire ctDNAs from the three *Oryza* species were constructed.

Sequencing of DNA

Fragments of DNA that contained deletions were subcloned into the appropriate sites of pUC119. Single-stranded DNAs were obtained by infection of helper phage M13K07 (Vieira and Messing 1987). Sequencing was carried out on an automated DNA sequencer (Applied Biosystems).

Synthesis of DNA and the polymerase chain reaction (PCR)

Synthesis of DNA and PCR were carried out using a DNA synthesizer (Applied Biosystems) and a DNA-Amplification System (Perkin-Elmer Cetus Corporation, USA), respectively.

Results

Restriction endonuclease analysis

The patterns of *Bam*HI restriction fragments from ctDNAs from four *Oryza* species are shown in Fig. 1. A large number of fragments were generated by this enzyme. The electrophoretic patterns of bands were similar, but three or four unique bands were detected in each species. Although these ctDNAs were relatively pure, with little contaminating nuclear and mitochondrial DNA, the amounts of ctDNA from *O. officinalis* and *O. australiensis* were not sufficient to allow detection of the fragments smaller than 1.5 kb.

Cloning of ctDNAs from O. punctata, O. officinalis, and O. australiensis

Since the yields of ctDNAs from the three *Oryza* species were too small to allow detailed analysis with restriction endonucleases, we constructed overlapping clone banks of the ctDNAs. To avoid the loss of ctDNAs as a result of size fractionation, we used the lambda FIX vector as described below.

The ctDNAs were partially digested with *Sau*3AI and "a partial fill-in reaction" was carried out. These ctDNAs were ligated separately with "lambda FIX/*Xho*I-partial fill-in treated DNA." "Partial fill-in reacted ctDNAs" cannot be ligated with one another but only with lambda FIX vector (Zabarovsky and Allikmets 1986). Recombinant phages were selected by plaque hybridization with ctDNA clones from *O. sativa* as probes. In order to

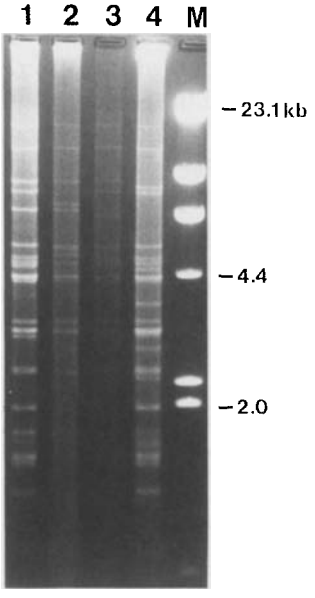

Fig. 1. Electrophoretic separation of *Bam*HI restriction fragments of ctDNAs. *1*, *O. sativa* (AA genome type); *2*, *O. australiensis* (EE); *3*, *O. officinalis* (CC); *4*, *O. punctata* (BB); *M*, lambda DNA digested with *Hin*dIII

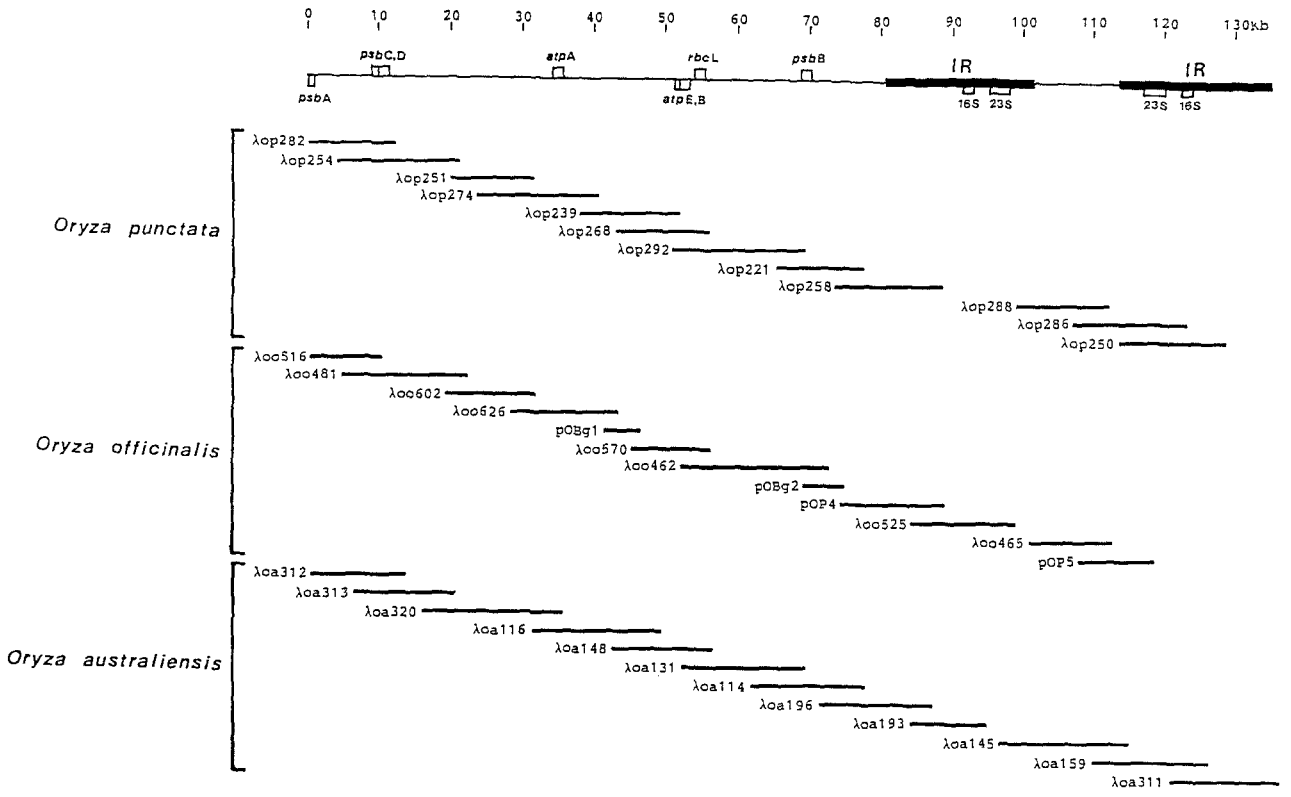

Fig. 2. Banks of overlapping clones of ctDNAs from *O. punctata*, *O. officinalis*, and *O. australiensis*. The extent of the inverted repeats (IR) and the positions of the genes for 23S rRNA (23S), 16S rRNA (16S), the large subunit of RuBPCase (*rbcL*), the α, β, and ε subunits of ATP synthase (*atpA*, *atpB*, *atpE*), and photosystem II complex (*psbA*, *psbB*, *psbC*,*psbD*) are indicated. Almost all clones are lambda phage clones, but the four clones of ctDNA from *O. officinalis* (pOBg1, pOBg2, pOP4, pOP5), are plasmid clones

obtain further clones, ctDNA from *O. officinalis* was completely digested with *Pst*I and *Bgl*II, and fragments were cloned into the *Pst*I and *Bam*HI sites of pUC19, respectively. Consequently, we obtained overlapping clones of ctDNAs from the three *Oryza* species (Fig. 2).

Comparison of physical maps between ctDNAs from four species of Oryza

The physical maps of recombinant phages and plasmids were determined by restriction endonuclease analysis and Southern hybridization with clones of ctDNA from *O. sativa* as probes. Connecting the maps of each clone, we were able to construct physical maps of the entire ctDNAs from the three *Oryza* species, and we compared these to that of *O. sativa* (Fig. 3). There are two types of variation, presumed to be mutations, among them. There are gains and losses of restriction sites. If the physical map of ctDNA from *O. sativa* is taken as the standard, the ctDNAs from *O. punctata*, *O. officinalis*, and *O. australiensis* have five, three, and eight site variations, respectively. The other type of variation was a deletion or insertion of nucleotides. Compared with that of *O. sativa*, the *Bam*HI-3/*Pst*I-3 fragment of *O. punctata* ctDNA was

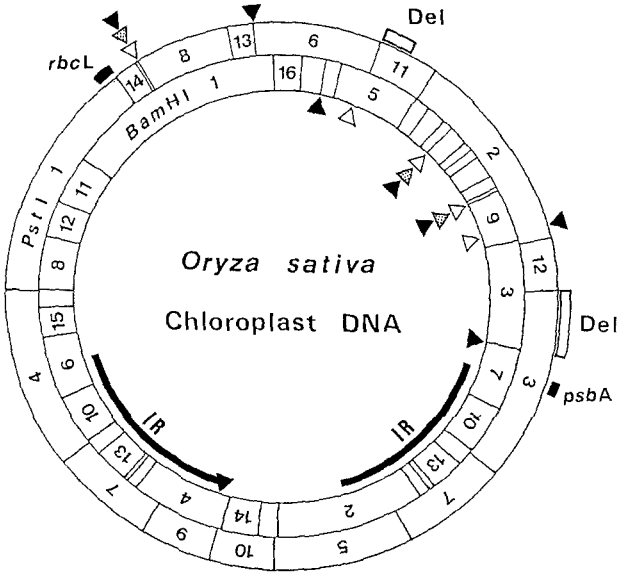

Fig. 3. The restriction map of ctDNA from *O. sativa* (*Pst*I and *Bam*HI), compared with those of *O. punctata*, *O. officinalis*, and *O. australiensis*. The notation "Del" with a rectangle and the triangles indicate deletions and restriction-site variations, respectively. White, gray, and black symbols refer to *O. punctata*, *O. officinalis*, and *O. australiensis*, respectively. The positions of the genes for *rbcL* and *psbA* are also shown

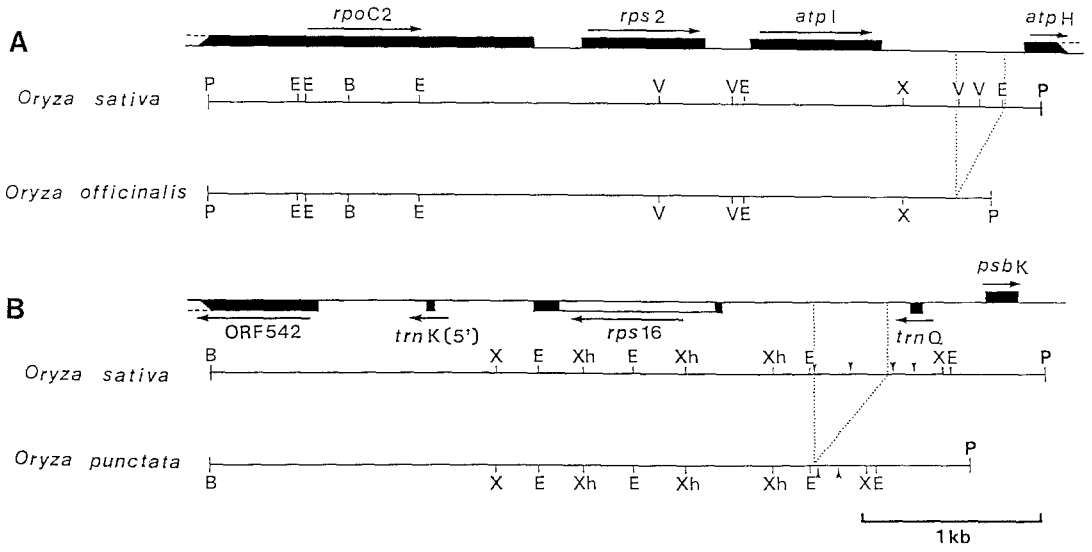

Fig. 4A, B. Comparison of deletions in ctDNAs of *O. officinalis* (**A**) and *O. punctata* (**B**), to that of *O. sativa*. The positions of the genes and the directions of transcription are shown. The symbols for the restriction sites are as follows: P, *Pst*I; E, *Eco*RI; B, *Bam*HI; V, *Eco*RV; X, *Xba*I; Xh, *Xho*I; and the small *triangles* (between E-X fragment) refer to *Taq*I. *Dotted lines* indicate deletions

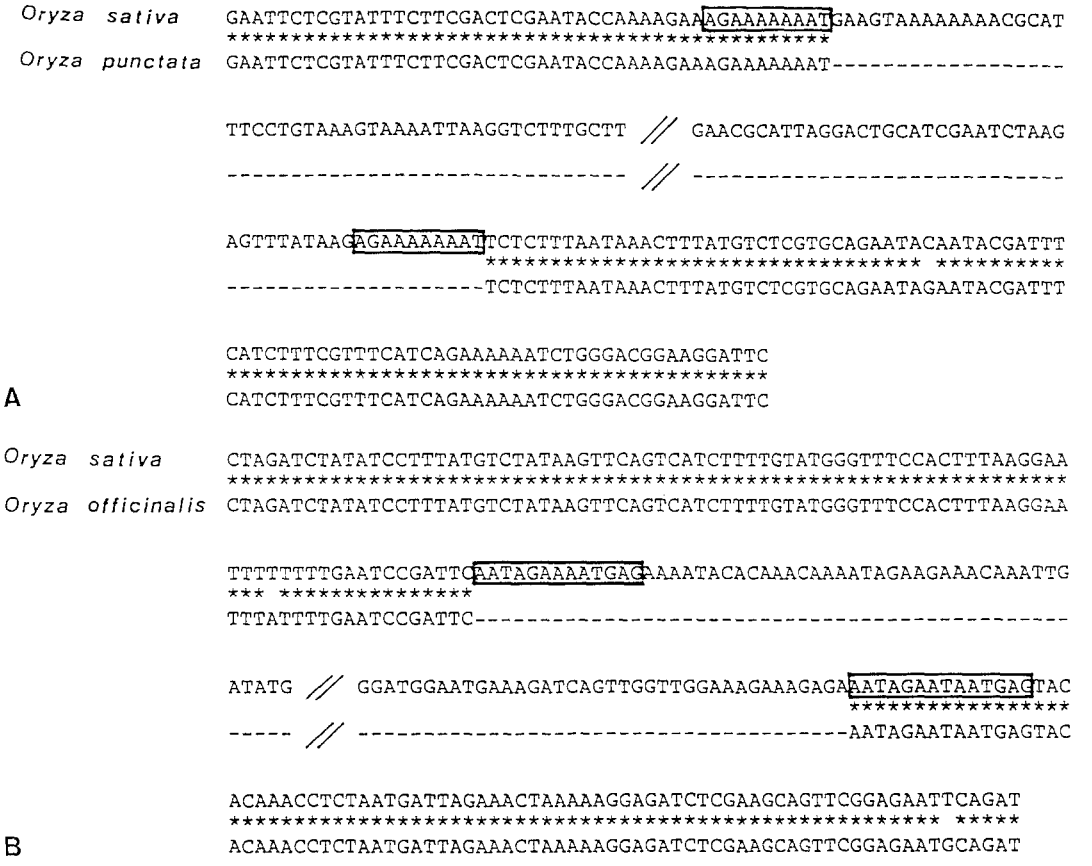

Fig. 5A, B. Comparison of the nucleotide sequence alignments around the deletions, between *O. sativa* and *O. punctata* (**A**) and between *O. sativa* and *O. officinalis* (**B**). *Stars* denote matching seuqences and *dashes* indicate gaps (deleted regions). The direct repeats that are considered to be sites of recombination are *boxed*

several hundred base pairs smaller. The *Bam*HI-5/*Pst*I-11 fragment of ctDNA from *O. officinalis* was also several hundred base pairs smaller than the corresponding fragment of *O. sativa*. However, we could not detect any deletions or insertions of more than 100 bp in the ctDNA from *O. australiensis*.

Nucleotide sequences around the deletions

To detect the precise locations of the deletions found in ctDNAs from *O. punctata* and *O. officinalis*, fragments of DNA that contained deletions were subcloned into plasmid vector pUC19. Restriction endonuclease analyses, with electrophoresis on agarose and polyacrylamide gels, and Southern hybridization with appropriate probes were carried out (data not shown). The deletion in ctDNA from *O. punctata* was located in an *Eco*RI-*Xba*I fragment (0.7 kb), between the *trnQ* gene and the *rps16* gene, and that in the ctDNA of *O. officinalis* was located in an *Xba*I-*Pst*I fragment (0.8 kb), between the *atpI* gene and the *atpH* gene (Fig. 4). These two fragments, which contained deletions, were subcloned into the appropriate sites of pUC119 and the nucleotide sequences were determined as described in 'Materials and methods.'

DNA sequences around the deletions were aligned for *O. sativa* and *O. punctata* (Fig. 5A), and for *O. sativa* and *O. officinalis* (Fig. 5B). Regions upstream and downstream from these deletion-containing regions exhibited a high degree of homology (>95%). This region in *O. punctata* was 453 bp shorter than that in *O. sativa*, and that in *O. officinalis* was 322 bp shorter than that in *O. sativa*. The deleted sequences had no homology. At the borders of the deleted regions, we detected direct repeats, AGAAAAAAAT and AATAGAA(T)AATGAG, in *O. sativa* at locations where we found the deletions in *O. punctata* and *O. officinalis*, respectively. They both had AT-rich sequences, but no homology was evident between them. This indicates that these deletions were a result of intramolecular recombination mediated by these direct-repeat sequences. This mechanism has been shown in wheat species (Ogihara et al. 1988).

Which Oryza species contain the deletions found in O. punctata and O. officinalis?

To examine whether other or not species of *Oryza* contain the deletions found in *O. punctata* (W1515) and *O. officinalis* (W0002), total DNAs were prepared from 15 *Oryza* species (30 strains, Table 1). Total DNAs were digested with *Xba*I and subjected to electrophoresis on agarose gels. To search for occurrence of the same deletion as was found in *O. punctata*, Southern hybridization was carried out using the 2.5-kb *Xba*I-*Xba*I fragment from *O. sativa* in which the deletion in *O. punctata* was found (Probe 1) and the PCR product that corresponded to the deleted sequence (Probe 2). The band patterns showed that there are deletions in *O. punctata* (W1514, W1515) and *O. minuta* (W1319, Y0022), because they generated bands that were about 400 bp smaller than the others, and their total DNAs did not hybridize with the deleted sequence (Fig. 6). To reveal distribution of the same deletion as was found in *O. officinalis*, the 4.7-kb *Pst*I fragment from *O. sativa* (Probe 3), in which the deletion in *O. officinalis* was found, and the PCR product that corresponded to the deleted sequence (Probe 4) were used as probes. The band patterns showed that there are the same deletions in *O. punctata* (W1564), *O. officinalis* (W0002, W0065) and *O. eichingeri* (W1521). The ctDNAs from three other *O. officinalis* strains (W0012, W0564, W1252) also contained the same deletion (data not shown).

Table 1. List of the *Oryza* species used for isolation of total DNA. Del(p) and Del(o) indicate carriers of deletions found in *O. punctata* (W1515) and *O. officinalis* (W0002), respectively

Species	Strain	Genome type	Deletion
O. sativa	Nipponbare	AA	
O. rufipogon	W0107	AA	
(annual type)	W0630	AA	
O. rufipogon	W0120	AA	
(perennial type)	W0149	AA	
O. meridionalis	W1298	AA	
	W1300	AA	
	W1625	AA	
O. glumaepatula	W1192	AA	
O. barthii	W0822	AA	
	W1467	AA	
O. glaberrima	W0025	AA	
O. longistaminata	W1477	AA	
O. punctata	W1514	BB	Del(p)
	W1515	BB	Del(p)
O. punctata	W1564	BBCC	Del(o)
O. minuta	W1319	BBCC	Del(p)
	Y0022	BBCC	Del(p)
O. officinalis	W0002	CC	Del(o)
	W0012	CC	Del(o)
	W0065	CC	Del(o)
	W0564	CC	Del(o)
	W1252	CC	Del(o)
O. eichingeri	W1521	CC	Del(o)
O. latifolia	W0542	CCDD	
	W1175	CCDD	
O. alta	W0017	CCDD	
O. grandiglumis	W1194	CCDD	
O. australiensis	W0008	EE	
	W1538	EE	

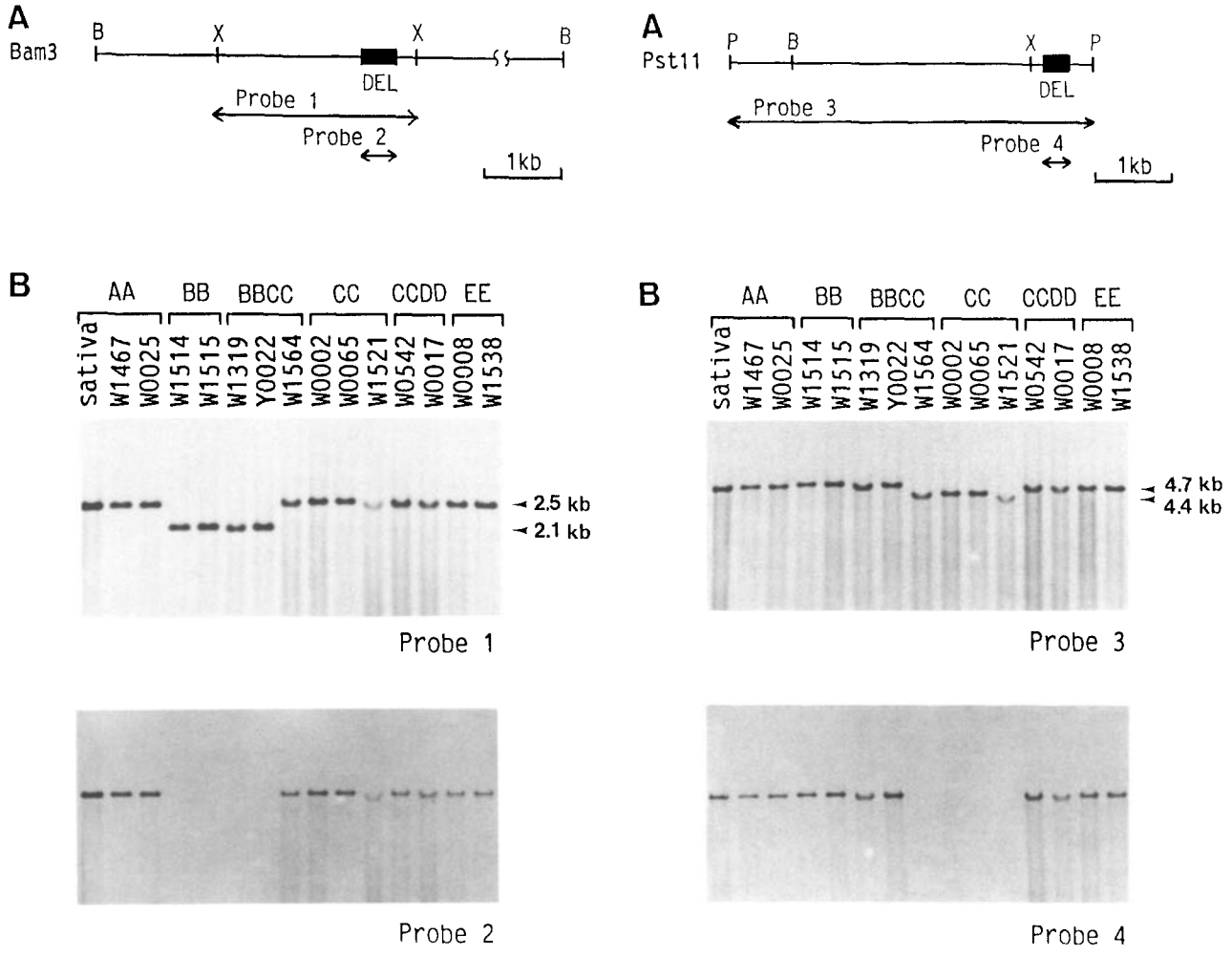

Fig. 6 A, B. Screening of the deletion found in *O. punctata* (W1515) among 15 *Oryza* strains. **A** Physical map of the *Bam*HI-3 fragment and the position of the deletion found in *O. punctata* (DEL). Restriction sites are shown for *Bam*HI (B) and *Xba*I (X). *Arrows* indicate fragments of DNA used as probes. **B** Southern hybridizations of Probes 1 and 2 to *Xba*I digests of total DNAs from 15 strains *Oryza* having different genome types

Fig. 7 A, B. Screening the deletion found in *O. officinalis* (W0002) among 15 *Oryza* strains. **A** Physical map of the *Pst*I-11 fragment and the position of the deletion found in *O. officinalis* (DEL). Restriction sites are shown for *Pst*I (P), *Bam*HI (B), and *Xba*I (X). *Arrows* indicate fragments of DNA used as probes. **B** Southern hybridizations of Probes 3 and 4 to *Pst*I digests of total DNA from 15 *Oryza* strains having different genome types

Discussion

Comparison of ctDNAs from four Oryza species

The *Bam*HI fragment patterns (Fig. 1) and the physical maps of ctDNAs from four *Oryza* species (Fig. 3) were similar but, nonetheless, distinguishable. There are some gains and losses of *Bam*HI and *Pst*I sites. These variations are assumed to be the result of base substitutions. Other variations found in *O. punctata* and *O. officinalis* are associated with the fragment sizes which are changed as a result of deletions or insertions. Since we detected the size variations by restriction endonuclease analysis on 0.7% agarose gels, it is likely that all deletions or insertions of more than 100 bp were detected. There are seven genome types of *Oryza*: AA, BB, BBCC, CC, CCDD, EE, and FF. Although we analyzed four of them, i.e., AA, BB, CC, and EE, we detected only two deletions/insertions among them. Thus, the number of deletions/insertions observed among *Oryza* species is much smaller than that reported in *Beta* and wheat (Kishima et al. 1987; Ogihara and Tsunewaki 1988). With respect to the diversity of ctDNAs, *Oryza* species are closer to one another than those of *Beta* and wheat (*Triticum* and *Aegilops*, inclusively).

As shown in Fig. 3, all the mutations found in ctDNAs among the four *Oryza* species in their ctDNAs are located between the *rbcL* and *psbA* genes in the large single-copy region. The results suggest that this region is

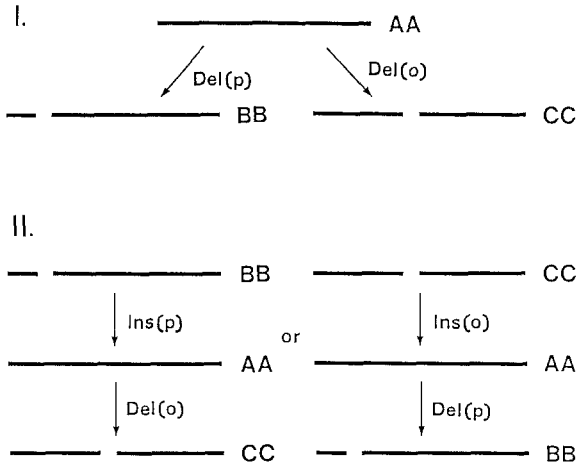

Fig. 8. Two cases for the phylogeny of ctDNAs in *Oryza*. AA, BB, and CC indicated the genome types of *Oryza* species. Del(p) and Del(o) indicate deleted fragments. Conversely, insertions of Del(p) and Del(o) are indicated by Ins(p) and Ins(o), respectively

highly variable evolutionarily in the ctDNAs of *Oryza* species, similar to ctDNAs of the *Nicotiana* species (Kung et al. 1982).

The deletions found in ctDNAs from O. punctata and O. officinalis are genome-specific variations

As shown in Table 1, the deletion in *O. punctata* (W1515, BB genome type) was found in another strain of *O. punctata* (W1514, BB). Similarly, the deletion in *O. officinalis* (CC genome type) was found in two species with the CC genome type, *O. officinalis* (W0002, W0012, W0065, W0564, W1252) and *O. eichingeri* (W1521). This suggests that the deletions found in *O. punctata* and *O. officinalis* are genome-specific variations.

In the BBCC genome type, there are two types of deletions: the BB genome-specific deletion as found in *O. punctata* (W1564) and the CC genome-specific deletion as found in *O. minuta* (W1319, Y0022). The BBCC genome type is considered to have originated as a result of crossing between the BB and CC genome types. Our evidence clearly indicates that there are diphyletic origins of the BBCC genome type, i.e., from the reciprocal crosses between the BB and CC genome types. The geographical distributions of *O. minuta* (BBCC) and *O. punctata* (BBCC) are The Philippines and Africa, respectively (Tateoka 1963; Tateoka and Pancho 1963). This supports diphyletic origins of the BBCC genome type.

The phylogenetic relationships among the three types found in Oryza

We found that two fragments of ctDNAs from *O. punctata* and *O. officinalis* were shorter than the corresponding fragments from *O. sativa*. How were these deletions/insertions produced during the evolution of the various species of *Oryza*? We have no evidence to answer this question at present. However, if we assume that mutational events had occurred at minimal times and that deletions had been hardly reversible, then two cases can be considered.

Case I: Deletion of the Del(p) and Del(o) fragments from the ctDNA of *O. sativa* (AA genome type) produced the ctDNAs of *O. punctata* (BB) and *O. officinalis* (CC), respectively. Del(p) and Del(o) correspond to the sequences with deletions found in ctDNAs from *O. punctata* and *O. officinalis*, respectively.

Case II: Insertion of Ins(p) into the ctDNA of BB produced the AA genome type and a subsequent deletion, Del(o), produced the CC genome type. On the other hand, insertion of Ins(o) into the ctDNA of CC produced the AA genome type and the subsequent deletion, Del(p), produced the BB genome type.

Both deletion and insertion are the results of DNA recombination. However, deletion, as caused by intramolecular recombination, seemed to occur more easily than insertion as caused by intermolecular recombination. Furthermore, Dally and Second (1990) reported that the genetic distance between the BB and CC genome types is closer than that between any other pair of genome types after a phylogenetic study with restriction endonuclease analysis. Thus, we prefer case I for the interpretation of the phylogeny of ctDNAs in *Oryza*.

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