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Comparative study on the chloroplast, mitochondrial and nuclear genome differentiation in two cultivated rice species, *Oryza sativa* and *O. glaberrima*, by RFLP analyses

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Summary. Restriction fragment length polymorphisms of chloroplast (ct), mitochondrial (mt) and nuclear DNA were investigated using eight cultivars of *Oryza sativa* and two cultivars of *O. glaberrima*. Relative variability in the nuclear and cytoplasmic genomes was estimated by a common measure, genetic distance. Based on the average genetic distances among ten cultivars for each genome, the evolutionary variabilities of the mitochondrial and nuclear genomes were found to be almost the same, whereas the variability of the chloroplast genome was less than half that of the other two genomes. Cluster analyses on ct and mt DNA variations revealed that chloroplast and mitochondrial genomes were conservative within a taxon and that their differentiations were well-paralleled with respect to each other. For nuclear DNA variation, an array of different degrees of differentiation was observed in *O. sativa*, in contrast with little variation in *O. glaberrima*. As a whole, differentiation between *O. sativa* and *O. glaberrima* was clearly observed in all three genomes. In *O. sativa*, no notable difference was found between the cultivars 'Japonica' and 'Javanica', whereas a large differentiation was noticed between 'Japonica' (including 'Javanica') and 'Indica'. In all three genomes, the average genetic distances within 'Indica' were much larger than those within 'Japonica' (including 'Javanica'), and almost similar between 'Japonica' (including 'Javanica') and 'Indica'. These facts indicate that differentiation in *O. sativa* was due mainly to 'Indica'.

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Key words: Cultivated rice species – Chloroplast DNA – Mitochondrial DNA – Nuclear DNA – RFLP analysis

Introduction

Three organelles in a plant cell contain genetic information: nucleus, chloroplast and mitochondrion. Genetic information in the nucleus is inherited from both parents, whereas that of chloroplasts and mitochondria are usually inherited maternally though with some exceptions such as *Oenothera*, alfalfa, and conifers (Neale et al. 1986). Although genomes in all three organelles are autonomous and independent in inheritance, they interact with each other in terms of gene expression.

Analysis of DNA variation during the last two decades has proved to be useful in elucidating the genetic differentiation and the phylogenetic relationships between related taxa. Pental and Barnes (1985) demonstrated differentiation among A-genome diploid species of rice using repetitive sequences of nuclear DNA. Zhao et al. (1989) also analyzed *Oryza* species with genome-specific repetitive sequence of nuclear DNA. Their results suggested that repetitive sequences can be used as molecular markers at the species or genome level. Wang and Tanksley (1989) used single-copy DNA probes and measured genetic variation in *O. sativa*. Chloroplast (ct) DNA variation has also been studied. Ichikawa et al. (1986) examined genetic relationships among species having different genome constitutions, while Ishii et al. (1986, 1988) analyzed different accessions of A-genome diploid species to

determine phylogenetic relationships. Furthermore, a large-scale survey covering 13 species (247 accessions) was made by Dally and Second (1990), who concluded that ctDNA analysis is a very powerful means of studying phylogenetic relationships. As for mitochondrial (mt) DNA, Kadowaki et al. (1988) showed polymorphism of plasmid-like DNA in *O. sativa*; however, only limited information is available on mtDNA variation in rice.

The above results of analyses on the DNA variation in the three different organelles can not be compared with each other because different materials were used in each analysis. Therefore, in this study, we have compared nuclear, chloroplast and mitochondrial genome differentiation at the DNA level, using the same accessions of two cultivated rice species.

Materials and methods

Plant materials

Eight *O. sativa* and two *O. glaberrima* cultivars were used for DNA isolation (Table 1). The *O. sativa* accessions included two cultivars each from 'Japonica' and 'Javanica', and four from 'Indica'.

DNA isolation

Chloroplast DNA was isolated from three-leaf-stage seedlings according to the method of Ishii et al. (1988). Purified ctDNA was used for the restriction endonuclease analysis.

Mitochondrial DNA was prepared by a modification of the procedure of Kemble et al. (1980) as follows: two-leaf-stage etiolated seedlings were homogenized in a Warning blender with Buffer A [0.44 M mannitol, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% BSA]. The homogenate was filtered through four layers of cheesecloth and two layers of miracloth. The suspension was subjected to differential centrifugation to collect crude mitochondria. The pellet was resuspended in Buffer G [0.3 M sucrose, 50 mM Tris-HCl (pH 8.0)], and treated with DNase. After incubation, intact mitochondria were obtained by washing three times with Shelf Buffer [0.6 M sucrose, 10 mM Tris-HCl (pH 8.0), 20 mM EDTA]. Purified mtDNA was used for the restriction endonuclease analysis.

Nuclear DNA was isolated from two-leaf-stage etiolated leaves by the method of Watson and Thompson (1986) after slight modification; a discontinuous gradient [30–45% Percoll, 0.5 M hexylenglycol, 10 mM Pipes-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5% Triton X-100] was used for nucleus purification, and the band at the 30–45% Percoll interface was collected. Purified nuclear DNA was used as the source of the probe DNA for Southern hybridization.

Total DNA was extracted by a modification of the method of Mettler (1987). Three-leaf-stage seedlings were homogenized quickly in liquid nitrogen. The leaf power was mixed with Extraction Buffer [1% N-lauroylsarcosine Na-salt, 0.25 M sucrose, 50 mM NaCl, 20 mM EDTA, 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol], and incubated at room temperature for 30 min. Protein of the mixture was removed by phenol extraction, followed by phenol:chloroform extraction. Total DNA was recovered by ethanol precipitation and resuspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. After RNase treatment, DNA was recovered again by ethanol precipitation. Purified total DNA was used for Southern-hybridization analysis of nuclear DNA.

Restriction endonuclease analysis

Chloroplast DNA was digested with three (*Eco*RI, *Hin*dIII and *Pst*I), and mtDNA with five (*Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II and *Xho*I), restriction endonucleases. All endonucleases used here are 6-bp cutters. Digestion was carried out according to the supplier's instructions (Takara Shuzo Co. Ltd., Japan, and Nippon Gene Co. Ltd., Japan). Digested ctDNA was electrophoresed with an 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA). Digested mtDNA was electrophoresed with both 0.6% and 1.2% agarose gels to get good separation of the high and low molecular-weight fragments, respectively. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) and photographed with UV-light illumination.

Cloning nuclear DNA fragments

Nuclear DNAs from *O. sativa* cv Nipponbare and IR36 were cleaved with *Hin*dIII or *Pst*I and inserted into plasmid pUC119. Eighteen independent clones were obtained and were used as probes for Southern-hybridization analysis of nuclear DNA.

Southern hybridization

Total DNA was digested with *Hin*dIII or *Pst*I and electrophoresed (5–10 μg per lane) with a 1.0% agarose gel. Transfer of the DNA fragments to Nylon membrane (Hybond-N, Amersham, UK) was made bidirectionally after Maniatis et al. (1982). Cloned nuclear DNAs were used as probes. They were labeled with non-radioactive digoxigenin-dUTP using a DNA Labeling and Detection Kit (Boehringer Mannheim, Germany). The membrane was hybridized overnight with the probe in the hybridization solution (5 × SSC, 0.5% Blocking reagent, 0.1% N-lauroylsarcosine Na-salt, 0.02% SDS) at 68 °C. The membrane was washed twice in 2 × SSC and 0.1% SDS solution at room temperature for 5 min, and twice in 0.1 × SSC and 0.1% SDS solution at 68 °C for 15 min. Immunological detection was done overnight according to the supplier's instructions (Boehringer Mannheim, Germany).

Table 1. Materials used as the source of chloroplast, mitochondrial and nuclear DNA

Taxon[a]	Code	Cultivar
O. sativa ecosp. 'Japonica'	J1	Nipponbare
O. sativa ecosp. 'Japonica'	J2	T65
O. sativa ecosp. 'Javanica'	Jv1	532
O. sativa ecosp. 'Javanica'	Jv2	647
O. sativa ecosp. 'Indica'	I1	419
O. sativa ecosp. 'Indica'	I2	C8005
O. sativa ecosp. 'Indica'	I3	108
O. sativa ecosp. 'Indica'	I4	IR36
O. glaberrima	G1	W401
O. glaberrima	G2	W528

[a] Classification after Takahashi (1984)

Estimation of genetic distance and the construction of phylogenetic trees

In the case of chloroplast and mitochondrial genomes, the numbers of total and common fragments of ten cultivars, were scored for the restriction fragment patterns of ctDNA and mtDNA obtained by treatment with three and five endonucleases, respectively. In the case of nuclear genomes, the numbers of total and common fragments were scored for the same ten rice cultivars in terms of the Southern hybridization patterns of total DNA obtained by combinations of two endonucleases and 18 nuclear DNA clones used as probes.

In all cases, the proportion of common fragments was calculated by the formula, $F_{ij} = 2B_{ij}/A_{ij}$, where A_{ij} and B_{ij} are the numbers of total and common fragments observed between the i^{th} and j^{th} cultivars (Nei and Li 1979). Using this value, the genetic distance between two cultivars was estimated according to Nei (1987).

A phylogenetic tree for each of the chloroplast, mitochondrial and nuclear genomes of the ten cultivars was constructed by the UPGMA method (Sneath and Sokal 1973), which was applied to the genetic distances obtained by the above procedures. In the case of the chloroplast genomes, a phylogenetic tree was constructed by the same method but in terms of the number of ctDNA mutations.

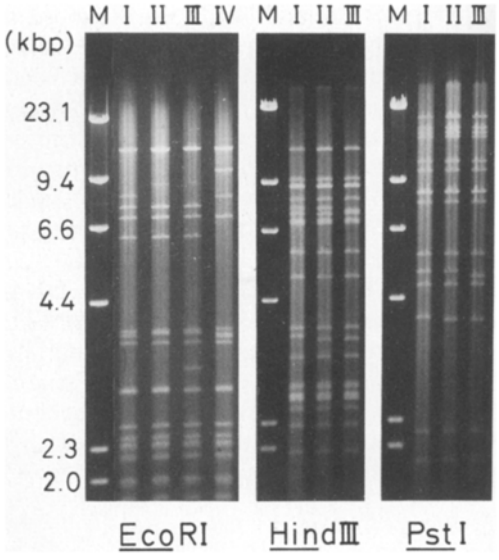

Fig. 1. *Eco*RI, *Hin*dIII and *Pst*I restriction fragment patterns of ctDNA observed among ten cultivars of *O. sativa* and *O. glaberrima*. *M*, lambda DNA digested with *Hin*dIII

Results

Restriction endonuclease analysis of ctDNA

Restriction fragment length polymorphisms (RFLPs) of ctDNA among ten cultivars were studied by restriction endonuclease analysis employing the following three endonucleases; *Eco*RI, *Hin*dIII and *Pst*I, all being 6-bp cutters. The *Eco*RI, *Hin*dIII and *Pst*I digests gave four (I–IV), three (I–III) and three (I–III) different patterns, respectively (Fig. 1). Table 2 shows the fragment constitutions, the molecular sizes of individual fragments, and the copy number observed in each pattern. Based on these fragment patterns, the chloroplast genomes of ten cultivars could be identified (Table 3) to be either of the four types, 1, 3, 4 and 5, previously reported by Ishii et al. (1988).

From the results given in Table 2, the numbers of both all, and the common, restriction fragments were calculated between every pair of the four chloroplast genomes, and the genetic distances were estimated (Table 4). Based on these data, clustering was worked out by the UPGMA method (Fig. 2A).

In order to clarify the nature of mutations existing among the four chloroplast genomes, Southern hybridization was employed. Using the P12 fragment as a probe, the changes in *Hin*dIII (H12) and *Pst*I (P12) fragments observed between types 1 and 3 were found to be caused by a common 0.1-kbp deletion in type 3. Similarly, using the P10 fragment as a probe, the changes in *Hin*dIII (H5) and *Pst*I (P10) fragments found between types 1 and 4 were ascribed to a common 0.2-kbp deletion in type 4. Furthermore, by

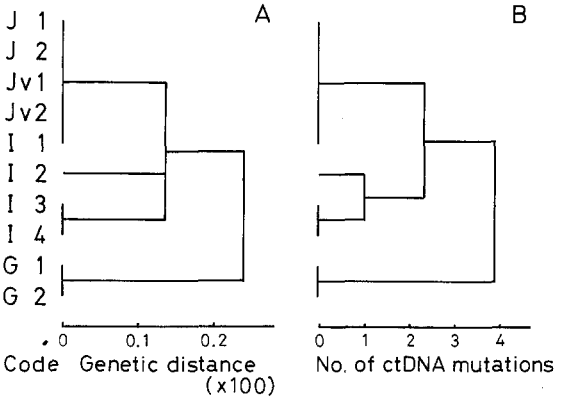

Fig. 2A, B. Dendrograms showing the genetic relationships between the chloroplast genomes of ten rice cultivars (indicated by code). **A** and **B** constructed respectively on Nei's genetic distances and the numbers of ctDNA mutations given in Table 4

physical mapping of the P10-H5 fragments of the type 1 and 4 chloroplast genomes, an *Eco*RI site was found to be lost by the same 0.2-kbp deletion in the type 4 genome (Fig. 3). Consequently, it also caused an *Eco*RI fragment change between the type 1 (2.9 + 0.4 kbp) and type 4 (3.1 kbp) genomes. Four other fragment changes have already been analyzed by Dally and Second (1990). Table 5 summarizes the nature of all the mutations found among the four chloroplast genome types. Based on the number of mutations detected between every pair of the four types (Table 4), a cluster analysis is given in Fig. 2B.

Table 2. Fragment constitutions and molecular sizes of the individual fragments identified in the electrophoretic patterns of three endonuclease digests of ctDNA from ten rice cultivars

*Eco*RI						*Hin*dIII					*Pst*I				
Fragment[a]		Type[b]				Fragment		Type			Fragment		Type		
No.	kbp	I	II	III	IV	No.	kbp	I	II	III	No.	kbp	I	II	III
E1	12.6	++	++	++	++	H1	12.9	+	+	+	P1	19.2	+	+	+
–	9.8	–	–	–	+	H2	9.6	+	+	+	P2	16.2	+	+	+
E2	7.4	+	+	+	–	H3	9.0	++	++	++	P3	15.2	+	+	+
E3	6.8	+	+	+	+	H4	8.2	+	+	+	P4	14.4	+	+	+
E4	6.1	+	+	+	–	H5	7.7	+	+	–	P5	10.9	+	+	+
–	3.9	–	–	–	+	–	7.5	–	–	+	P6	10.1	+	+	+
E5	3.8	+	+	+	–	H6	7.4	+	+	+	P7	8.4	++	++	++
–	3.8	–	+	+	–	H7	7.1	+	+	+	P8	7.8	+	+	+
E6	3.7	+	–	–	–	H8	6.9	+	+	+	P9	5.5	+	+	+
–	3.7	–	–	–	+	H9	5.8	+	+	+	P10	5.1	+	+	–
–	3.7	–	–	–	+	H10	5.0	+	+	+	–	4.9	–	–	+
E7	3.6	+	+	+	+	H11	3.8	+	+	+	P11	4.7	+	+	+
–	3.1	–	–	+	–	H12	3.6	+	–	–	P12	3.9	+	–	–
E8	2.9	+	+	–	+	–	3.5	–	+	+	–	3.8	–	+	+
E9	2.9	+	+	+	+	H13	3.2	+	+	+	P13	2.2	+	+	+
E10	2.9	+	+	+	+	H14	2.8	+	+	+	P14	1.8	+	+	+
E11	2.5	++	++	++	++	H15	2.8	+	+	+					
E12	2.4	+	+	+	+	H16	2.7	++	++	++					
E13	2.4	+	+	+	+	H17	2.6	++	++	++					
E14	2.3	++	++	++	++	H18	2.5	+	+	+					
E15	2.3	+	+	+	+	H19	2.3	+	+	+					
E16	2.2	+	+	+	+	H20	2.0	+	+	+					
E17	2.2	+	+	+	+										
E18	2.0	+	+	+	+										
E19	2.0	+	+	+	+										
E20	2.0	+	+	+	+										
E21	1.8	+	+	+	+										
E22	1.8	+	+	+	+										

[a] Fragment designation follows Hiratsuka et al. (1989) and Dally and Second (1990). –, new fragments not seen previously
[b] ++, + and –, double copy, single copy and no copy, respectively

Table 3. Four chloroplast genome types identified among ten rice cultivars with the *Eco*RI, *Hin*dIII and *Pst*I restriction fragment patterns of their ctDNAs

Ct genome type[a]	Rest. frag. pattern			Cultivar (code)
	*Eco*RI	*Hin*dIII	*Pst*I	
1	I	I	I	J1, J2, Jv1, Jv2, I1
3	II	II	II	I3, I4
4	III	III	III	I2
5	IV	I	I	G1, G2

[a] After Ishii et al. (1988)

Table 4. Number of the total and the common restriction fragments, genetic distances and the number of ctDNA mutations estimated between four chloroplast genomes of rice

Ct genome types compared	Total no. fragments	No. common fragments	Genetic distance (×100)	No. ctDNA mutations
1–3	126	60	0.272	2
1–4	126	57	0.561	3
1–5	126	59	0.366	3
3–4	126	60	0.272	1
3–5	126	57	0.561	5
4–5	126	54	0.867	6

Note: three 6-bp cutters, *Eco*RI, *Hin*dIII and *Pst*I, were used

Restriction endonuclease analysis of mtDNA

RFLPs of mtDNA among ten cultivars were studied by restriction endonuclease analysis using the 6-bp cutters *Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II and *Xho*I. Figure 4 shows the *Pst*I restriction fragment patterns obtained by 1.2% agarose gel electrophoresis. In each restriction pattern, the fragments larger than 2 kbp were scored. Based on the proportion of common restriction fragments observed between mtDNAs from the ten cultivars, the genetic distances were estimated (Table 6) and the clustering of these genomes is given in Fig. 5.

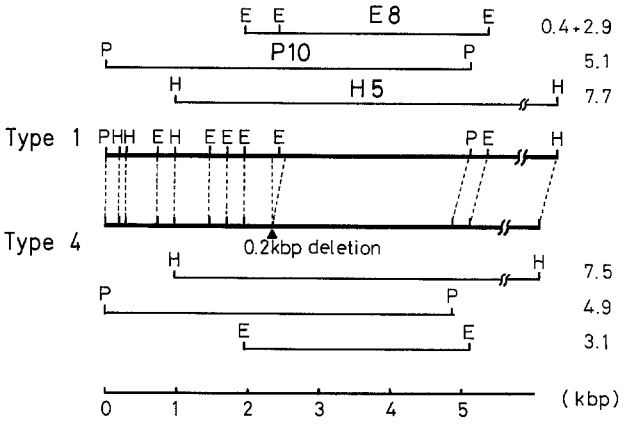

Fig. 3. Physical maps of the P10-H5 fragments in the type 1 and 4 chloroplast genomes, indicating a single 0.2-kbp deletion in type 4 causes the different sizes of fragments in *Eco*RI, *Hin*dIII and *Pst*I digestions. *E, H and P*, *Eco*RI, *Hin*dIII and *Pst*I sites, respectively

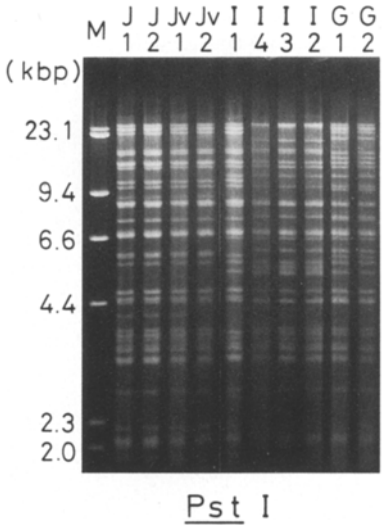

Fig. 4. *Pst*I restriction fragment patterns of mtDNA from ten rice cultivars. *M*, lambda DNA digested with *Hin*dIII

Table 5. Six ctDNA mutations found among four chloroplast genomes of rice

Mutation[a]	Mutant chloroplast genome type	Fragment involved	Fragment size (kbp)	
			Standard	Mutant
0.1-kbp insertion	3, 4	E6	3.7	3.8
0.1-kbp deletion	3, 4	H12	3.6	3.5
		P12	3.9	3.8
0.2-kbp deletion	4	E8	2.9 + 0.4	3.1
		H5	7.7	7.5
		P10	5.1	4.9
0.1-kbp insertion	5	E5	3.8	3.9
*Eco*RI site gain	5	E2	7.4	3.7 + 3.7
*Eco*RI site loss	5	E4, E6	6.1 + 3.7	9.8

[a] All mutations were characterized using the type 1 genome as a standard

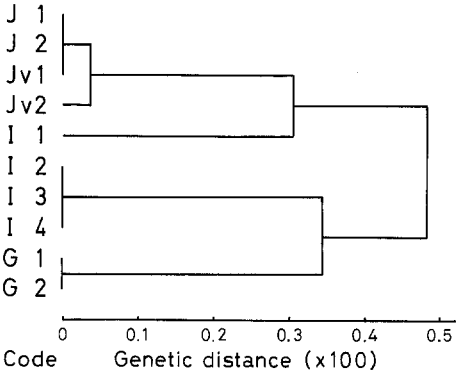

Fig. 5. A dendrogram showing the genetic relationships between the mitochondrial genomes of ten rice cultivars, based on the Nei's genetic distances given in Table 6

Table 6. The number of the total restriction fragments (above diagonal) and the genetic distances × 100 (below diagonal) between mtDNAs of ten rice cultivars

Code	J1	J2	Jv1	Jv2	I1	I2	I3	I4	G1	G2
J1	–	330	330	330	334	333	333	333	341	341
J2	0.000	–	330	330	334	333	333	333	341	341
Jv1	0.000	0.000	–	330	334	333	333	333	341	341
Jv2	0.067	0.067	0.067	–	334	333	333	333	341	341
I1	0.603	0.603	0.603	0.641	–	337	337	337	345	345
I2	1.016	1.016	1.016	1.016	0.841	–	336	336	343	343
I3	1.016	1.016	1.016	1.016	0.841	0.000	–	336	343	343
I4	1.016	1.016	1.016	1.016	0.841	0.000	0.000	–	343	343
G1	0.989	0.989	0.989	0.989	0.822	0.789	0.789	0.789	–	352
G2	0.989	0.989	0.989	0.989	0.822	0.789	0.789	0.789	0.000	–

Note: five 6-bp cutters, *Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II, and *Xho*I, were used

Table 7. Detection of RFLPs of the nuclear DNA among ten rice cultivars by Southern hybridization analysis using 18 nuclear DNA clones as probes

Nuclear DNA clone	Origin of clone[a]	Cloning site	Size (kbp)	Hybrid. pattern[b]	
				*Hin*dIII	*Pst*I
1	N	*Pst*I	1.0	P	M
2	N	*Pst*I	1.1	P	M
3	N	*Pst*I	1.4	P	M
4	N	*Pst*I	1.6	P	M
5	N	*Pst*I	2.5	M	M
6	N	*Pst*I	2.8	–	M
7	N	*Pst*I	3.7	P	P
8	N	*Pst*I	3.9	M	M
9	N	*Pst*I	4.8	M	M
10	N	*Pst*I	10.5	P	M
11	N	*Pst*I	12.6	M	M
12	I	*Hin*dIII	1.4	–	M
13	I	*Hin*dIII	3.1	–	M
14	I	*Hin*dIII	5.0	P	P
15	I	*Hin*dIII	5.2	P	P
16	I	*Pst*I	2.1	–	M
17	I	*Pst*I	2.7	P	M
18	I	*Pst*I	4.7	–	M

[a] N, 'Nipponbare' ('Japonica'); I, 'IR36' ('Indica')
[b] M, monomorphic; P, polymorphic; –, not tested

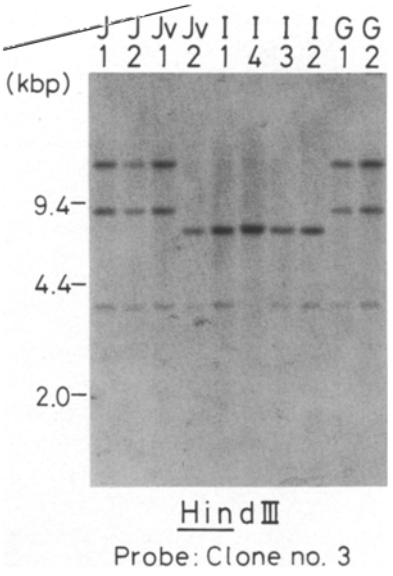

Fig. 6. Southern hybridization patterns of nuclear DNA clone no. 3 used as a probe to the *Hin*dIII-digested total DNAs of ten rice cultivars

Southern hybridization analysis of nuclear DNA

RFLPs of the nuclear DNA of the ten cultivars were studied by Southern-hybridization analysis. Two 6-bp cutters, *Hin*dIII and *Pst*I, were used for the total DNA digestion and 18 nuclear DNA clones were employed as probes. RFLPs were revealed by 12 out of the 31 enzyme-probe combinations tested (Table 7). Figure 6 shows an example of the polymorphic fragment pattern. All of the hybridized fragments in each cultivar were scored, and the numbers of the total and common fragments between all pairs of ten cultivars were calculated. Using these data, the genetic distances

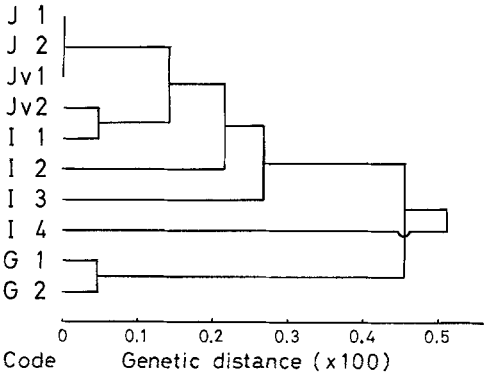

Fig. 7. A dendrogram showing the genetic relationships between the nuclear genomes of ten rice cultivars based on the Nei's genetic distances given in Table 8

Table 8. Number of the total Southern hybridized bands (above diagonal) and the genetic distances × 100 (below diagonal) between nuclear DNAs of ten rice cultivars

Code	J1	J2	Jv1	Jv2	I1	I2	I3	I4	G1	G2
J1	–	122	122	121	121	121	119	113	120	120
J2	0.000	–	122	121	121	121	119	113	120	120
Jv1	0.000	0.000	–	121	121	121	119	113	120	120
Jv2	0.233	0.233	0.233	–	120	120	118	112	119	119
I1	0.333	0.333	0.333	0.094	–	120	118	112	119	119
I2	0.534	0.534	0.534	0.286	0.286	–	118	112	119	119
I3	0.540	0.540	0.540	0.497	0.394	0.711	–	110	117	117
I4	1.163	1.163	1.163	0.868	0.868	0.750	0.647	–	111	111
G1	0.698	0.698	0.698	0.982	1.092	1.335	0.660	1.313	–	118
G2	0.802	0.802	0.802	0.982	1.092	1.335	0.769	1.313	0.095	–

Note: total DNA was treated with two 6-bp cutters (*Hin*dIII and *Pst*I) and probed with 18 nuclear DNA clones

between all cultivars were estimated (Table 8) and a dendrogram showing their genetic relationships is given in Fig. 7.

Discussion

Differentiation at the molecular level of each of the chloroplast, mitochondrial and nuclear genomes among ten rice cultivars

In several crops and their relatives, such as *Brassica* (Palmer et al. 1983), maize (Doebley et al. 1987) and wheat (Ogihara and Tsunewaki 1988), the phylogenetic relationships among different chloroplast genomes have been analyzed based on the number of ctDNA mutations observed between them. However, it is still difficult to analyze mitochondrial and nuclear genome differentiation by this method. Consequently, the proportions of common restriction fragments or else the genetic distances between different DNA samples have been used to evaluate mitochondrial and nuclear genome differentiation at the DNA level; examples are the mitochondrial genomes of *Triticum* and *Aegilops* (Terachi and Tsunewaki 1986) and the nuclear genomes of *Brassica* (Song et al. 1988) and *Oryza* (Wang and Tanksley 1989). In the present study, genetic distances were used to compare chloroplast, mitochondrial and nuclear genome differentiation. In addition, the numbers of ctDNA mutations were employed to analyze chloroplast genome differentiation.

The chloroplast genomes of ten rice cultivars were classified into four types. The phylogenetic relationships between these four types were based on the genetic distances between chloroplast genomes and on the numbers of ctDNA mutations. They agree with each other, except for one 'Indica' cultivar (I2) which has a type 4 chloroplast genome. Based on genetic distance (Fig. 2A), this chloroplast genome clustered first with those of other *O. sativa* cultivars, whereas it was closely related to the type 3 chloroplast genome of cultivars I3 and I4 when the numbers of ctDNA mutations were considered (Fig. 2B). This is mainly due to a 0.2-kbp deletion in the type 4 chloroplast genome that affected three different restriction fragment patterns (see Fig. 3 and Table 5). For this reason, a phylogenetic tree based on the number of ctDNA mutations seems to be a better reflection of the real relationships among the four chloroplast genomes. On this basis, they can be grouped into three main clusters; two 'Japonica', two 'Javanica' and one 'Indica' cultivar (J1, J2, Jv1, Jv2 and I1), three 'Indica' cultivars (I2, I3 and I4), and two of *O. glaberrima* (G1 and G2). The predominant chloroplast genome types in each group, i.e., types 1, 3 and 5, respectively, have already been found among wild species accessions (Ishii et al. 1988). This indicates strong conservatism of the chloroplast genome during the evolution of cultivated rice species equivalent to that also reported in other crops, such as wheat (Ogihara and Tsunewaki 1982, 1988) and maize (Doebley et al. 1987).

Mitochondrial genomes were classified into five types (Fig. 5). These were grouped into four main clusters, one consisting of 'Japonica' and 'Javanica' cultivars (J1, J2, Jv1 and Jv2), two of 'Indica' (I1; I2, I3 and I4), and one of *O. glaberrima* (G1 and G2). Notable mitochondrial genome differentiation was present among them with much less, or else no, differentiation within each group. Except for one cluster consisting of 'Indica' cultivar I1, the three other main clusters are the same as those of the chloroplast genomes (Fig. 2B). This coincidence seems to reflect the co-evolution between chloroplast and mitochondrial genomes through maternal inheritance.

Nuclear genomes were classified into eight types. No differences were found among two 'Japonica' and one 'Javanica' cultivar (J1, J2 and Jv1), as was also the case for their chloroplast and mitochondrial genomes. Small differences were found between two *O. glaberrima* cultivars (G1 and G2), which showed no differences in their chloroplast and mitochondrial genomes. The nuclear genomes of four 'Indica' cultivars (I1, I2, I3 and I4) differed greatly from each other and from those of 'Japonica' and *O. glaberrima*, although the chloroplast genome of I1 was identical to that of the 'Japonica' cultivars, and the mitochondrial genomes of I2, I3 and I4 were identical to each other. As a whole, the nuclear genomes of *O. sativa* and *O. glaberrima* are differentiated from each other; in contrast, in *O. sativa* an array of different degrees of differentiation was revealed with rather discrete differentiation in their chloroplast and mitochondrial genomes.

Comparison between the chloroplast, mitochondrial and nuclear genome differentiation

The relative variability of the three kinds of genomes among the ten cultivars of the two *Oryza* species was estimated by genetic distance. The average genetic distances among these cultivars were 0.305×10^{-2} for chloroplast genomes, 0.703×10^{-2} for mitochondrial genomes and 0.651×10^{-2} for nuclear genomes (Table 9). This parameter is known to be independent of genome size, and gives an estimation of the rate of base change per site. Therefore, we can conclude that the evolutionary variabilities of the mitochondrial and nuclear genomes are almost the same as each other, whereas the variability of the chloroplast genome is less than half that of the mitochondrial and nuclear genomes. It is well known that the DNA sequences forming the coding or regulatory regions are highly conserved in evolution, compared to those of spacer

Table 9. Average genetic distances within and between taxa based on the chloroplast, mitochondrial and nuclear genome differentiation

Genetic distance	Total no. compared	Average genetic distance (× 100)		
		CtDNA	MtDNA	NucDNA
Within all cultivars	45	0.305	0.703	0.651
Within *O. sativa*	28	0.217	0.620	0.493
Within 'Japonica' (including 'Javanica')	6	0.000	0.034	0.117
Within 'Indica'	6	0.275	0.421	0.609
Within *O. glaberrima*	1	0.000	0.000	0.095
Between *O. sativa* and *O. glaberrima*	16	0.477	0.893	0.961
Between 'Japonica' and 'Indica'	16	0.276	0.915	0.591
Between 'Japonica' and *O. glaberrima*	8	0.366	0.989	0.808
Between 'Indica' and *O. glaberrima*	8	0.589	0.797	1.114

regions. In higher plants, the proportion of the spacer regions to the entire genome is largest in the nuclear genome, followed by the mitochondrial genome, and is least in the chloroplast genome. The conservative nature of the chloroplast genome is well explained by this high proportion of coding sequences and their uniparental transmission. However, the observed high variability of the mitochondrial genome, which is comparable to that of the nuclear genome, cannot be explained in this way. The mitochondrial genome undergoes frequent intra- as well as inter-molecular recombination, thus existing as a heterogeneous molecular species (Sederoff 1987). This fact suggests that plant mitochondrial genomes have not developed through an orderly system of DNA replication and transmission as has the evolution of the nuclear genome. Rather it has involved a high rate of nucleotide divergence per site.

The present finding of the high evolutionary variability of the mitochondrial genome in rice is in contrast to the results of Wolfe et al. (1987) who reported that the mutation rate of the mitochondrial genome is lower than those of the other two genomes. They compared various nucleotide sequences among ct, mt and nuclear DNAs, and concluded that the silent (synonymous) substitution rate in mtDNA is less than one-third that in ctDNA, and not more than one-fifth that in nuclear DNA. The discrepancy between our findings and their results on the evolutionary divergence of the mitochondrial genome in comparison with those of the chloroplast and nuclear genomes might be attributable to the difference in the materials and regions investigated. Wolfe et al. (1987) examined DNA sequences in coding and non-coding regions between widely separated species, such as wheat and maize, or even between monocot and dicot species, whereas we dealt with the restriction fragments generated from whole regions of the genome between rice cultivars having the same nuclear, AA, genome. It is not known, however, how the phylogenetic distance between two taxa affects the relative evolutionary variabilities between chloroplast, mitochondrial and nuclear genome.

Inter- and intra-specific differentiation in cultivated rice species

Based on the results of chloroplast, mitochondrial and nuclear DNA analyses, average genetic distances within and between taxa were calculated (Table 9). As no notable differences were found between 'Japonica' and 'Javanica' in their chloroplast, mitochondrial and nuclear genomes, two 'Japonica' and two 'Javanica' cultivars were treated as 'Japonica' for calculating average genetic distances. Average genetic distances within 'Indica' were very large in all three genomes as compared with those within 'Japonica' (including 'Javanica') and within *O. glaberrima*. This indicates that 'Indica' contained very large variation in all three genomes. Differentiation between *O. sativa* and *O. glaberrima* was clearly observed, because genetic distances between these two were much greater than those within each of them. Genetic distances between 'Japonica' and 'Indica' gave more or less similar values with those within 'Indica', and much higher than those within 'Japonica'. These facts indicate that the differentiation between 'Japonica' and 'Indica' occurred in all three genomes, whereas differentiation within *O. sativa* was mainly due to 'Indica'.

The differentiation observed in cultivated species seems to reflect their origins from different wild progenitors. Oka (1974, 1988) and Morishima et al. (1963) proposed that *O. sativa* and *O. glaberrima* had originated from the Asian form of *O. perennis* and *O. breviligulata*, respectively. In fact, ctDNA analysis of these wild species supported their proposal and indicated conservatism of ctDNA in cultivated forms (Ishii et al. 1988). However, information on the genetic

variability in wild species at the DNA level is still limited to the chloroplast genome. There is a need to study mtDNA and nuclear DNA variation in wild species which are related to cultivated species of the genus *Oryza* in order to establish phylogenetic relationships between wild and cultivated species.

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