CYTOPLASMIC AND NUCLEAR GENOME DIFFERENTIATION IN A-GENOME DIPLOID SPECIES OF RICE AS REVEALED BY THE RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF DNAS

TAKASHIGE ISHII

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CONTENTS

I. INTRODUCTION

The genus Oryza includes two cultivated species and about 20 wild species (Tateoka 1963). The ground work on their genome analysis was carried out by Morinaga (1964), followed by Katayama (1967), Watanabe and Ono (1965, 1967) and many others. It is generally agreed that the genomes A and C are basic and that the genomes B, C and D show some affinity for each other. Two cultivated species, O. sativa and O. glaberrima, and some wild species form a group, having the genome A in common.

The classification and nomenclature of wild A-genome spesies are somewhat confusing because of their richness in variation. Morishima et al. (1961) classified them into O. perennis and O. breviligulata. O. perennis is used for a wild taxon with long ligule, and O. breviligulata for African wild taxon with short and round ligule. As O. perennis distributes widely, Morishima (1969) furthermore divided it into four geographical forms, Asian, American, African and Oceanian. In this study, their classification and nomenclature are used.

As for cultivated species, O. sativa and O. glaberrima are closely related to O. perennis and O. breviligulata, respectively. O. sativa has long ligule as O. perennis does, and O. glaberrima has short and round ligule like O. breviligulata. O. glaberrima is locally cultivated only in Africa, whereas O. sativa is widely cultivated in the tropical and temperate zones of the world.

Under the variable environmental condition, O. sativa shows broad genetic differentiation. Kato and Maruyama (1928) noticed the existence of two groups in this species. They classified them into two types, "Japonica" and "Indica" types, based on morphology and sexual affinity (Kato et al. 1928). Matsuo (1952) investigated 22 external morphological characters of several thousand varieties and recognized three main plant types, A, B and C. The centers of their distribution are Japan, Java and India, respectively. Oka (1958) examined 12 morphological and physiological traits of 120 varieties, and classified them into two groups, Continental and Insular groups. Insular group was further subdivided into Tropical-Insular and Temperate-Insular. These names by

themselves indicate their geographical distribution. From all these studies on morphological and physiological traits, O. sativa varieties are reasonably classified into three main groups, Japonica, Javanica and Indica. Takahashi (1984) suggested that they correspond to ecospecies, a term used by an ecologist Turesson (1922). Conceptional relationship between ecospecies, ecotype and ecophene is given in Fig. 1. Takahashi ranked the three types recognized by Matsuo (1952) and Oka (1958) to the ecospecies, and named Japonica, Javanica and Indica. In this study, his classification is used.

Besides the studies on morphological traits, other approaches have been made to clarify the intra- and interspecific variation in A-genome diploid species. Biochmical studies, such as isozyme analysis, have provided useful information. Nakagahra et al. (1975, 1977) revealed continuous geographic cline based on allelic frequencies of O. sativa esterase isozymes. Second (1982) examined 40 presumed isozyme loci and found that most O. sativa cultivars formed two clusters, corresponding to Japonica and Indica. The large-scale survey on isozyme polymorphism in O. sativa by Glaszmann (1985, 1987) led him to classify O. sativa into six varietal groups, i.e., two major (corresponding to Japonica and Indica), two minor and two satellite groups. Furthermore, Second (1985, 1986) extended the analysis to the wild species. In view of the similarity of isozyme patterns, he suggested that Indica and Japonica cultivars were derived from South Asian and Chinese O. rufipogon (= O. perennis Asian form), respectively.

Recently, analysis of DNA variation has been shown to be very useful in elucidating the differentiation and the phylogenetic relationships between related taxa. In rice, Pental and Barnes (1985) showed the differentiation in A-genome diploid species using repetitive sequences of nuclear DNA. Zhao et al. (1989) analyzed Oryza species with genome-specific repetitive sequences of nuclear DNA. Their results suggest that the repetitive sequences can be used as molecular markers at the species or genome level. Wang and Tanksley (1989) used single-copy nuclear DNAs as probes to measure the degree of genetic variation in O. sativa. They could classify 70 accessions using ten probes. Cordesse et al. (1990) studied variation of the intergenic spacer region of ribosomal DNA in A-genome diploid species, and the results were found to be in

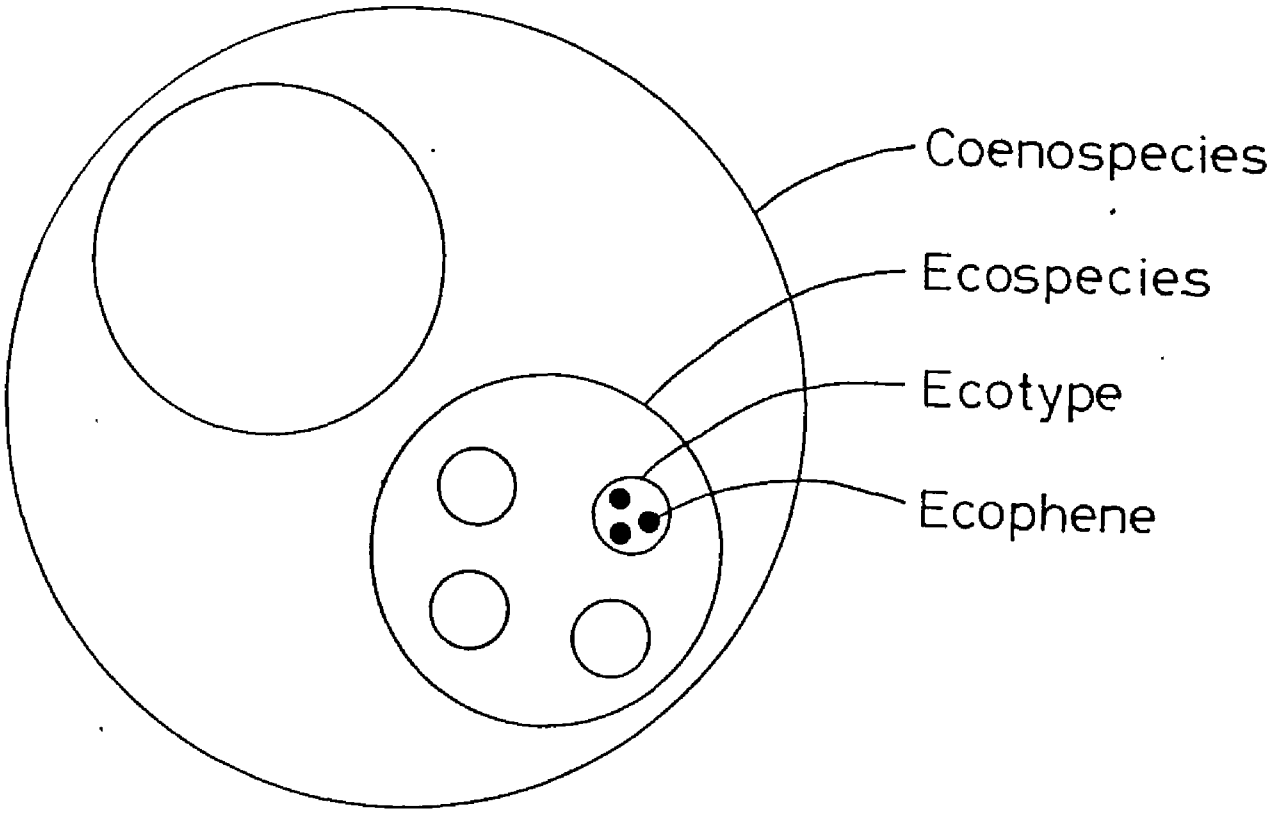


Fig. 1. Turesson's scheme showing the relationships among coenospecies, ecospecies, ecotype and ecophene.

good agreement with the data obtained by isozyme analysis. Further, Sano and Sano (1990) analyzed the same variation and suggested that high variability in ribosomal DNA could be a good clue to clarify the evolutionary divergence within species.

Especially, restriction endonuclease analysis of chloroplast (ct) and mitochondrial (mt) DNA was successfully applied for several higher-plant taxa, such as Triticum and Aegilops (Vedel et al. 1978; Ogihara and Tsunewaki 1982, 1988; Terachi et al. 1984), Zea (Timothy et al. 1979), Oenothera subsection Euoenothera (Gordon et al. 1982), Brassica (Palmer et al. 1983), Solanum (Hosaka 1986) and Avena (Murai and Tsunewaki 1987). In rice, after Hirai et al. (1985) constructed the first physical map of ctDNA, Ichikawa et al. (1986) and Ishii et al. (1986, 1988) identified interspecific relationships between species having different genomes and A-genome diploid species, respectively. Furthermore, a large-scale survey was done by Daily and Second (1990), concluding that ctDNA analysis is a very powerful means of studying phylogenetic relationships. As for mtDNA, Kadowaki et al. (1988) showed polymorphism of plasmid-like DNA in O. sativa, but there is no systematic research so far made on mtDNA variation.

In the present investigation, the author intended to clarify the phylogenetic relationships among A-genome diploid species and to elucidate the variation in cultivated rice species based on the chloroplast, mitochondrial and nuclear genome differentiation revealed by the restriction fragment length polymorphism of their DNAs. The following three studies (A-C) were carried out for this purpose.

A. Chloroplast genome differentiation in A-genome diploid species: Restriction endonuclease analysis on ctDNA was carried out in order to clarify phylogenetic relationships between chloroplast genomes of cultivated and wild A-genome diploid species. Further, a large number of local cultivars were examined to elucidate intraspecific ctDNA variation in O. sativa.

B. Mitochondrial genome differentiation between O. sativa and O. glaberrima: MtDNA was studied by restriction endonuclease and Southern hybridization analysis. This study aimed at revealing mitochondrial genome differentiation in cultivated rice species.

C. Nuclear genome differentiation in A-genome diploid species: Nuclear DNAs from four species were subjected to Southern hybridization analysis in order to carry out phylogenetic study on nuclear genome in A-genome diploid species.

II. MATERIALS AND METHODS

A. Materials

A large number of cultivars or accessions from two cultivated and two wild Oryza species, all having the genome A in common, were used. These are O. sativa, O. glaberrima, O. perennis and O. breviligulata. The first two are cultivated, and the last two are wild species. The following four groups of the materials were employed in various studies with different aims:

Group 1, consisting of 33 cultivars of O. sativa, eight cultivars of O. glaberrima, 21 accessions of O. perennis, and four accessions of O. breviligulata (Table 1). .

Group 2, containing 15 cultivars of O. sativa, and five cultivars of O. glaberrima (Table 2).

Group 3, including 115 cultivars of O. sativa collected from 17 Asian countries (Table 3).

Group 4, consisting of eight cultivars of O. sativa, six cultivars of O. glaberrima, 13 accessions of O. perennis, and three accessions of O. breviligulata (Table 4).

All accessions of Group 1 and 2 were used for ctDNA analysis. Further, ten cultivars of Group 2 were selected for mt and nuclear DNA analysis. In Group 3, ct and nuclear DNAs from 75 and 112 out of 115 cultivars were examined, respectively. Most of the accession in Group 4 were analyzed on both their ct and nuclear DNAs, however, four accessions of O. perennis were studied only on their nuclear DNAs.

Table 1. Materials used as the source of ctDNA

Taxon	Cultivar	Source[1]	Origin[2]
O. sativa ecosp. Japonica	Nipponbare	H	Japan
" "	Norin No.22	H	Japan
" "	Taichung No. 65	S	Taiwan
" "	Toride No. 1	P	Japan
" "	Homarenishiki	P	Japan
" "	Aichiasahi	P	Japan
" "	Yamabiko	P	Japan
" "	Akebono	Y	Japan
" "	Iwaimochi	Y	Japan
" "	501	S	Japan
" "	563	S	Japan
O. sativa ecosp. Javanica	242	S	Philippines
" "	532	S	Japan
" "	647	S	Philippines
" "	(C5810) Taichung No. 65[3]	S	Indonesia
" "	(C5811) Taichung No. 65[3]	S	Indonesia
O. sativa ecosp. Indica	101	S	Taiwan
" "	104	S	Taiwan
" "	108	S	Taiwan
" "	115	S	Taiwan
" "	144	S	Taiwan
" "	414	S	India
" "	415	S	India
" "	419	S	India
" "	435	S	Sri Lanka
" "	437	S	India
" "	444	S	India
" "	(868) Taichung No. 65[3]	S	Taiwan
" "	1034	S	China
" "	C5444	S	India
" "	C8005	S	India
" "	IR36	T	---
" "	Twx1	Y	---
O. glaberrima	(W025) Taichung No. 65[3]	S	---
"	W401	Y	Guinea
"	W438	S	---
"	(W440) Fujiminori[3]	Y	Guinea
"	W446	S	Guinea
"	W492	S	---
"	W528	S	Guinea
"	gl	Y	---

Table 1. (continued)

Taxon	Accession	Source[1]	Origin[2]
O. perennis Asian form	(W107) Taichung No. 65[3]	S	India
" "	W108	S	India
" "	W120	S	India
" "	W149	S	India
" "	W593	S	Malaysia
" "	W630	S	Burma
" "	W1943	S	China
" "	W1945	S	China
" "	W2028	S	Indonesia
" American form	W1167	S	Cuba
" "	W1169	S	Cuba
" "	W1185	S	Surinam
" "	W1186	S	Surinam
" "	W1192	S	Brazil
" African form	W1414	S	Sierra Leone
" "	W1460	S	Benin
" "	W1504	S	Tanzania
" "	W1540	S	Congo
" "	W1608	S	Nigeria
" Oceanian form	W1299	S	Australia
" "	W1633	S	Australia
O. breviligulata	W607	S	Guinea
"	W653	S	Sierra Leone
"	W720	S	Sudan
"	W1152	S	---

1) H: T. Horie, S: Y. Sano, P: Y. Peng, Y: T. Yabuno, T: S. Tsuji. Their affiliated institutions are given in the text.
2) --- : Unknown
3) Alloplasmic line of Taichung No. 65 or Fujiminori having the cytoplasm of the accession indicated in the parentheses. They were classified according to their cytoplasm donor.

Table 2. Materials used as the source of chloroplast, mitochondrial and nuclear DNA

Code	Taxon	Cultivar
J 1	O. sativa ecosp. Japonica	Nipponbare
J 2	" "	Taichung No. 65
J 3	" "	Norin No. 22
J 4	" "	Aichiasahi
J 5	" "	501
Jv1	" ecosp. Javanica	532
Jv2	" "	647
Jv3	" "	(C5810) Taichung No. 65[1)]
Jv4	" "	(C5811) Taichung No. 65[1)]
I 1	" ecosp. Indica	419
I 2	" "	C8005
I 3	" "	108
I 4	" "	IR36
I 5	" "	104
I 6	" "	C5444
G 1	O. glaberrima	W401
G 2	"	W528
G 3	"	W438
G 4	"	(W025) Taichung No. 65[1)]
G 5	"	(W440) Fujiminori[1)]

1) Alloplasmic line of Taichung No. 65 or Fujiminori having the cytoplasm of the accession indicated in the parentheses.
They were classified according to their cytoplasm donor.

Table 3. Materials used as the source of chloroplast and nuclear DNA in O. sativa

No.	Acc.No[1)]	Name	Enzymatic Group[2)]	Origin
1	---	IR36	--	Unknown
2	328	Azucena	VI	Philippines
3	1107	Ta Hung Ku	VI	China
4	1112	Hei Chiao Chui Li Hsiang Keng	VI	China
5	1254	Y Chang Ju	VI	China
6	3688	Dular	II	India
7	3717	N 32	II	India
8	5423	Salumpikit	I	Unknown
9	6046	DA 11	I	Bangladesh
10	6246	DA 28	II	Bangladesh
11	6264	N 22	II	India
12	6267	ASD 1	I	India
13	6274	PTB 9	I	India
14	6294	T 1	II	India
15	6304	PTB 30	II	India
16	6307	Jhona 349	II	India
17	6331	CO 18	I	India
18	6386	PTB 25	I	India
19	6422	DA 8	II	Bangladesh
20	6426	Basmati 370	V	Bangladesh
21	6538	Bamoia 341	III	Bangladesh
22	6541	Bhadoia 233	III	Bangladesh
23	6550	Birain 360	I	Bangladesh
24	7722	Madael	I	Sri Lanka
25	7755	Kalukantha	--	Sri Lanka
26	8896	S 624 (AC 398)	I	India
27	8952	Rathuwee	I	Sri Lanka
28	8972	Suduwee	--	Sri Lanka
29	9177	JC 91	I	India
30	12881	Dom-Zard	V	Iran
31	17052	Chuan 4	VI	Taiwan
32	17054	Haifugoya	VI	Taiwan
33	23364	Kinandang Patong	VI	Philippines
34	26276	Bikyat	VI	Philippines
35	27509	Baran Boro	II	Bangladesh
36	27513	Dholi Boro	II	Bangladesh
37	27516	Jagri Boro	II	Bangladesh
38	27536	Boteswar 2	II	Bangladesh
39	27590	Rayada 16-04	IV	Bangladesh
40	27762	Leuang Pratew	I	Thailand

1) Accession number of International Rice Research Institute, Philippines.
2) Enzymatic groups classified by Glaszmann (1985).

Table 3. (continued)

No.	Acc.No	Name	Enzymatic goup	Origin
41	27798	Basmati 1	V	Pakistan
42	30238	Champa Tong 54	I	Thailand
43	30342	Sulig	VI	Philippines
44	31525	Rathal	I	Sri Lanka
45	32292	Domsiah	V	Iran
46	32300	Gerdeh	II	Iran
47	32561	Dular	II	India
48	33187	Kaukkyi	V	Myanmar
49	33188	Kaukkyi Ani	--	Myanmar
50	33192	Kaukkyisau	V	Myanmar
51	33888	Yelaik Meedon	V	Myanmar
52	38698	NPE 844	--	Pakistan
53	40673	Pin Tawng	I	Thailand
54	43369	Cere Air	I	Indonesia
55	43372	Cicih Beton	VI	Indonesia
56	43394	Gogo Lempuk	VI	Indonesia
57	43400	Ilis Air	I	Indonesia
58	43530	Patik	I	Indonesia
59	43540	Pelita Janggut	I	Indonesia
60	43675	Trembese	VI	Indonesia
61	45624	DA 1	I	India
62	45975	Kalamkati	II	India
63	47529	Nep Cai Chiem 1	I	Vietnam
64	51064	Sinna Sithina Kali	I	Sri Lanka
65	51250	Ai-Chiao-Hong	I	China
66	51300	Guan-Yin-Tsan	I	China
67	51350	Lu-Lu-Tsan	I	China
68	51400	Pa-Tou-Hung	I	China
69	55457	Beonjo	VI	Korea
70	56036	Chau	I	Vietnam
71	58278	Ghati Kamma Nangarhar	II	Afghanistan
72	58881	Basmati Lamo	V	Nepal
73	58930	Chhote Dhan	V	Nepal
74	1154	Shan Kiu Ju	VI	China
75	1217	Ken Chiao Ju Hsiao Li	VI	China
76	5854	DA 9	I	Bangladesh
77	6102	CO 12	I	India
78	6245	DA 16	II	Bangladesh
79	6254	ADT 12	II	India
80	7718	Thahanala	--	Sri Lanka

Table 3. (continued)

No.	Acc.No	Name	Enzymatic group	Origin
81	8341	Dhola Aman (Lowland Aman)	I	Bangladesh
82	8946	Muthusamba	--	Sri Lanka
83	9176	JC 92	I	India
84	9178	JC 120	I	India
85	9179	JC 117	I	India
86	10214	Chiem Chanh	I	Vietnam
87	11640	Kap Nhay	VI	Laos
88	12880	Dom-Sofid	V	Iran
89	12883	Mehr	V	Iran
90	17051	Chuan 3	VI	Taiwan
91	20884	ARC 10372	II	India
92	23710	Dam	VI	Thailand
93	23729	Hawm Om	VI	Thailand
94	23754	Ma Hae	VI	Thailand
95	23764	Niaw Tew	I	Thailand
96	25867	Jhum Begunbichi	II	Bangladesh
97	26872	Binulawan	VI	Philippines
98	27519	Tepi Boro	II	Bangladesh
99	27588	Rayada 16-02	IV	Bangladesh
100	27591	Rayada 16-05	IV	Bangladesh
101	27630	Darmani	V	Nepal
102	27716	Kaw Luyoeng	I	Thailand
103	27869	Chahora 144	V	Pakistan
104	28924	Aus 61	II	Bangladesh
105	29726	Chaing Roneal	I	Kampuchea
106	32368	Tchampa	V	Iran
107	43322	Arang	I	Indonesia
108	43397	Gotak Gatik	VI	Indonesia
109	43434	Kenanga	I	Indonesia
110	43545	Popot	I	Indonesia
111	46768	T 26	V	India
112	53637	Basmati 217	V	India
113	25901	Miriti	II	Bangladesh
114	27748	Khao Dawk Mali 105	I	Thailand
115	39261	Firooz	V	Iran

Table 4. Materials used as the source of chloroplast and nuclear DNA

No.	Taxon	Accession
1	O. perennis Asian form	W108
2	" "	W120
3	" "	W149
4	" "	W593
5	" "	W630
6	" American form	W1167
7	" "	W1169
8	" "	W1192
9	" African form	W1618
10	" Oceanian form	W1300
11	" "	W1627
12	" "	W1629
13	" "	W1633
14	O. breviligulata	W607
15	"	W653
16	"	W1152
17	O. sativa ecosp. Indica	IR36
18	" ecosp. Japonica	Nipponbare
19	" "	Norin No.22
20	" ecosp. Javanica	532
21	" "	647
22	" ecosp. Indica	108
23	" "	419
24	" "	C8005
25	O. glaberrima	W401
26	"	W438
27	"	W440
28	"	W446
29	"	W492
30	"	W528

B. Methods

CtDNA extraction

CtDNA extraction was carried out after Tsunewaki and Ogihara (1983) and Enomoto et al. (1985). About 50 gm of seedling leaves were cut into 1 cm long, and homogenized in liquid nitorogen twice, each for 6 sec at 18,000 rpm using a homogenizer (Nissei AM-7, Nihonseiki Co. Ltd., Japan). After liquid nitorogen was completely evaporated, 500 ml of Buffer A (0.44 M mannitol; 50 mM Tris-HCl, pH 8.0; 3mM EDTA; 1 mM 2-mercaptoethanol; 0.1 % BSA) were added to the leaf powder and were homogenized three times with a home-use mixer. The suspension was filtered through four layers of cheesecloth and two layers of Miracloth. The filtrate was centrifuged for 5 min at 150 X g using an angle rotor to remove the debris. Once again the supernatant was centrifuged for 10 min at 1,900 X g using an angle rotor. The pellet was suspended in 7.5 ml of Buffer A (excluding mannitol), and centrifuged for 30 min at 112,500 X g using a swing rotor in a sucrose discontinuous gradient (15, 40 and 60 %) made with Buffer A. The green band formed at the 40 - 60 % interface was collected, and gradually diluted with 2 volumes of Buffer B (0.44 M mannitol; 50 mM Tris-HCl, pH 8.0; 3 mM EDTA). Intact chloroplasts were isolated by centrifugation for 10 min at 1500 X g using an angle rotor.

The pellet of intact chloroplasts was suspended in 2 ml of TE buffer (50 mM Tris-HCl, pH 8.0; 20 mM EDTA), and lysed in 0.5 ml of TE buffer containing 10 % sodium N-lauroyl sarcosinate. To this solution 0.5 mg of Proteinase K (Merck Co. Ltd., Germany) was added, and kept at 37 ^{o}C for 1 hr. From this preparation, ctDNA was extracted by a modified method of Kolodner and Tewari (1975), as follows. The chloroplast sample was made up to 3.7 ml by adding TE buffer, to which 3.7 gm of sterilized CsCl was gently dissolved. After adding ethidium bromide (200 µg/ml at the final concentration), the mixture was centrifuged for 9 hr at 240,000 X g using a vertical rotor. The fluorescent fraction with the UV light was collected, and ethidium bromide was removed by gentle shaking with TE-CsCl solution which was saturated with isopropyl alcohol. CtDNA was

recovered from the solution by ethanol precipitation and suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

MtDNA extraction

MtDNA was prepared by a modification of the procedure of Kemble et al. (1980) as follows. Two-leaf-stage etiolated seedlings were homogenized in Waring blender with Buffer A. 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. Intact mitochondria were obtained by washing the solution three times with Shelf Buffer (0.6 M sucrose; 10 mM Tris-HCl, pH 8.0; 20 mM EDTA). MtDNA was extracted from the intact mitochondria suspension by the same method as used for ctDNA extraction.

Nuclear DNA extraction

Nuclear DNA was isolated from two-leaf-stage etiolated leaves by the method of Watson and Thompson (1986) after slight modification; rice nuclei were purified using a discontinuous gradient (30 and 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), and the band formed at the 30 - 45 % interface was collected. Nuclear DNA was recovered from the intact nuleus suspension by the same method as used for ctDNA extraction.

Total DNA extraction

Total DNA was extracted by a modification of the method of Mettler (1987). Five to seven leaves were collected and homogenized quickly in liquid nitrogen with mortar and pestle. The leaf powder was mixed with Extraction Buffer (1 % sarkosyl; 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 temparature for 30 min. Protein of the mixture was removed by phenol extraction, followed by phenol and

chloroform:isoamylalcohol 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, purified DNA was recovered by ethanol precipitation.

Restriction endonuclease analysis

The following 11 endonucleases were used; BamHI, BglII, EcoRI, EcoRV, HindIII, PstI, PvuII, SalI, SmaI, XbaI and XhoI. Ct, mt and total DNAs were digested with the restriction endonucleases selected from them. Digestion was carried out according to the supplier's instructions (Takara Shuzo Co. Ltd., Japan, and Nippon Gene Co. Ltd., Japan). Digested ct and total DNAs were electrophoresed using 0.8 % and 1.2 % agarose gel in TAE buffer (40 mM Tris-acetate; 2 mM EDTA), respectively. Digested mtDNA was electrophoresed using both 0.6 % and 1.2 % agarose gels to get good separation of 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. Restriction fragments were analyzed based on their molecular weight.

Cloning ctDNA fragments

CtDNAs from O. sativa ecosp. Japonica cv. Nipponbare, ecosp. Indica cv. IR36, and O. glaberrima strain W401 were cleaved with restriction endonuclease PstI and inserted into the PstI site of a plasmid pUC119 with T4 DNA ligase under the conditions recommended by the supplier (Takara Shuzo Co. Ltd., Japan). The plasmid pUC119 contains an ampicillin-resistant gene, one PstI site in lacZ gene and intergenic region of phage M13. Before ligation, this plasmid was treated with bacterial alkaline phosphatase (Takara Shuzo Co. Ltd., Japan) to prevent self-ligation.

The recombinant plasmids were used to transform cells of Escherichia coli strain JM109, according to the transformation protocol of Frozed storage III (Hanahan 1985). The transformed cells were spread onto LB-plate containing 50 μg/ml of ampicillin, 40 μl of X-gal (20 mg/ml) and 10 μl of IPTG (1M), and were incubated overnight at 37 ^{o}C. E. coli cells which carry recombinant

plasmids can be detected from the colony color. Because of the insertion of ctDNA fragment into the lacZ gene, they can not produce blue colored bromochloroindole from X-gal and consequently form white colonies. The isolation of plasmids was carried out after the alkaline lysis method (Maniatis et al. 1982; Sambrook et al. 1989).

Cloned ctDNA fragments, of which designation follows to Hiratsuka et al. (1989), are as follows: From cv. Nipponbare – P1, P4, P5, P6, P7, P8, P9, P10, P11, P13 and P14 fragments, corresponding to about 73 % of total ctDNA. From cv. IR36 – P7, P8, P11, P13 and P14 fragments, corresponding to about 25 % of the total ctDNA. And, from strain W401 – P6, P8, P10, P11, P13 and P14 fragments, corresponding to about 23 % of the total ctDNA. They were used as probes in Southern hybridization or for sequencing analysis.

Cloning nuclear DNA fragments

Nuclear DNAs of O. sativa cv. Nipponbare and IR36 were cleaved with HindIII or PstI and inserted into the plasmid pUC119. Clonig procedure is the same as used for ctDNA fragment cloning except for the E. coli strain of competent cells. In order to achieve high efficiency of transformation, E. coli strain DH5α was used instead of strain JM109. As a result, 18 independent clones were obtained.

Probe DNA preparation

CtDNA fragments were prepared by cloning or gel recovery. Cloned ctDNA fragments described above and a plasmid pBR322 containing P12 fragment, which was kindly provided by D. A. Hirai, Nagoya University, Japan, were used as probes. Other probes of ctDNA fragments were recovered directly from agarose gel after electrophoresis using glass powder (GENECLEAN, BIO 101, Inc., USA).

The following four DNA fragments were used for Southern hybridization analysis of mtDNA; ATPA and COXII from pea (Morikami and Nakamura 1987), and RRN18&5 and RRN26 from wheat (Falconet et al. 1984, 1985).

For Southern hybridization analysis of nuclear DNA, 18 random-cloned and 12 single-copy nuclear DNA fragments were used as probes. Twelve single-copy DNAs were kindly provided by Dr. S. D. Tanksley, Cornell University, USA, and Dr. S. McCouch, International Rice Research Institute, Philippines.

Southern hybridization with radioactive probes

Probe DNA was labeled with [α-^{32}P] dCTP (400 Ci/mM, Amersham, UK) using Nick-Translation Kit (Takara Shuzo Co. Ltd., Japan). After electrophoresis, sample DNA in agarose gel was transferred to Nylon membrane (Biodyne A, Pall Ultrafine Filtration Co., USA).

Hybridization of the probe to the membrane was carried out overnight at 65 ^{o}C in the hybridization solution (5 X Denhardt; 5 X SSPE; 0.2 % SDS; 500 µg/ml salmon sperm DNA). The membrane was washed three times for 30 min at room temperature in the washing solution (5 mM sodium phosphate, pH 7.0; 1 mM EDTA; 0.2 % SDS), and autoradiographed with Fuji RX film and Fuji lightening-plus intensifying screen (Fuji Film Co. Ltd., Japan) at -70 ^{o}C.

This method was applied only for the ctDNA Southern hybridization in the study A.

Southern hybridization with non-radioactive probes

Probe DNA was labeled with non-radioactive Dig-dUTP using DNA Labeling and Detection Kit (Boehringer Mannheim, Germany). Transfer of the electrophoresed DNA (ct, mt and total DNA) fragments from the gel to Nylon membrane (Hybond-N, Amersham, UK) was made bidirectionally after Maniatis et al. (1982).

Hybridization was carried out as follows. The membrane was hybridized overnight with the probe in the Hybridization Solution (5 X SSC; 0.5 % Blocking reagent, 0.1 % N-lauroylsarcosine Na-salt; 0.02 % SDS) at 68 ^{o}C. The membrane was washed twice in 2 X SSC and 0.1 % SDS solution at room temperature for 5 min, and twice in 0.1 X SSC and 0.1 % SDS solution at 68 ^{o}C for 15 min. Immunological detection was done overnight, according to the supplier's instructions (Boehringer Mannheim, Germany). In case of Southern hybridization with single-copy nuclear

DNA as probe, labeling and detection were carried out after Ishii et al. (1990).

Sequence analysis

The PstI ctDNA fragments inserted into the plasmid pUC119 were used for sequence analysis. Single-stranded DNA was prepared according to the method of Terachi et al. (1987) using plasmid pUC119 and helperphage M13 K07 system (Takara Shuzo Co. Ltd., Japan), with the following modification: E. coli strain JM109 was used as a host for phage instead of strain MV1184. Sequencing reaction was carried out by dideoxy chain termination method (Sanger et al. 1977) with M13 Sequencing Kit (Takara Shuzo Co. Ltd., Japan). The single-stranded DNA was labeled with [α-^{32}P] dCTP (400 Ci/mM, Amersham, UK), and the gel was autoradiographed with Fuji RX film at room temperature.

Isozyme analysis

Isozyme polymorphisms were examined after Glaszmann et al. (1988). Young leaf tissue was homogenized with 0.5 % 2-mercaptoethanol. Filter paper was used to absorb the extract. They were inserted in 14 % starch gel containing System I buffer (9 mM Tris; 5 mM Histidine, pH 8.0). After electrophoresis, the gel was sliced and stained. In this experiment, the following five loci of two enzymes were analyzed; Pgi-1 and Pgi-2 of phosphoglucose isomerase (PGI), Amp-1, Amp-2 and Amp-3 of aminopeptidase (AMP). Staining solutions to detect the bands of these enzymes are as follows:

Phosphoglucose isomerase staining solution for Pgi-1 and Pgi-2

1 mg/ml fructose-6-phosphate; 0.2M Tris-HCl, pH 8.5; 4 mM $MgCl_2$; 0.1 mg/ml NADP; 0.2 U/ml Glucose-6-phosphate dehydrogenase; 0.2 mg/ml NBT; 0.02 mg/ml PMS; 1 % agar.

Alanine aminopeptidase stalning solution for Amp-1 and Amp-2

1 mg/ml DL-alanyl-β-naphthylamide; 0.3 mg/ml Fast black K salt; 0.1 M Tris-maleate, pH 3.3; 40 mM NaOH.

Leucine aminopeptidase staining solution for Amp-1 and Amp-3

0.5 mg/ml L-leucyl-β-naphthylamide; 0.3 mg/ml Fast black K salt; 0.1 M Tris-maleate, pH 3.3; 40 mM NaOH

III. RESULTS

A. Chloroplast genome differentiation in A-genome diploid species

Restiriction endonuclease analysis on ctDNA variation in four A-genome diploid species

In order to get a general picture on ctDNA variation in four A-genome diploid species, restriction endonuclease analysis on ctDNAs of 33 cultivars of O. sativa (11 Japonica, 5 Javanica and 17 Indica cultivars), eight cultivars of O. glaberrima, 21 accessions of O. perennis (9 Asian, 5 American, 5 African and 2 Oceanian accessions), and four accessions of O. breviligulata (Table 1) was carried out, using three restriction endonucleases, EcoRI, HindIII and PstI.

Their restriction fragment patterns were compared with each other. Seven EcoRI (type I-VII), three HindIII (type I-III) and four PstI (type I-IV) fragment patterns were found among 66 accessions, respectively (Figs. 2 and 3). Their fragment constitutions, including the molecular sizes of individual fragments, are given in Table 5. Table 6 summarized the ctDNA restriction fragment patterns of all accessions. Based on the EcoRI, HindIII and PstI restriction fragment patterns, chloroplast genomes (=ctDNA type) of the 66 accessions could be classified into nine types (1-9), and the number of the accessions of the four Oryza species showing the nine types is given in Table 7. Types 1, 3 and 5 were found in both the cultivated and wild species. O. sativa ecosp. Indica and the Asian and Amirican forms of O. perennis are polymorphic, each possessing three or four chloroplast genome types. In contrast, O. sativa ecosp. Japonica and Javanica, O. glaberrima and O. breviligulata were all found to be monomorphic.

Detailed studies on ctDNA variation in O. sativa and O. glaberrima

From O. sativa ecosp. Japonica, Javanica and Indica, and O. glaberrima, five (J1, J2, J3, J4 and J5), four (Jv1, Jv2, Jv3 and Jv4), five (I1, I3, I4, I5 and I6) and five (G1, G2, G3, G4 and G5) cultivars were selected, respectively, as shown in Table 2. Their ctDNAs were subjected to further

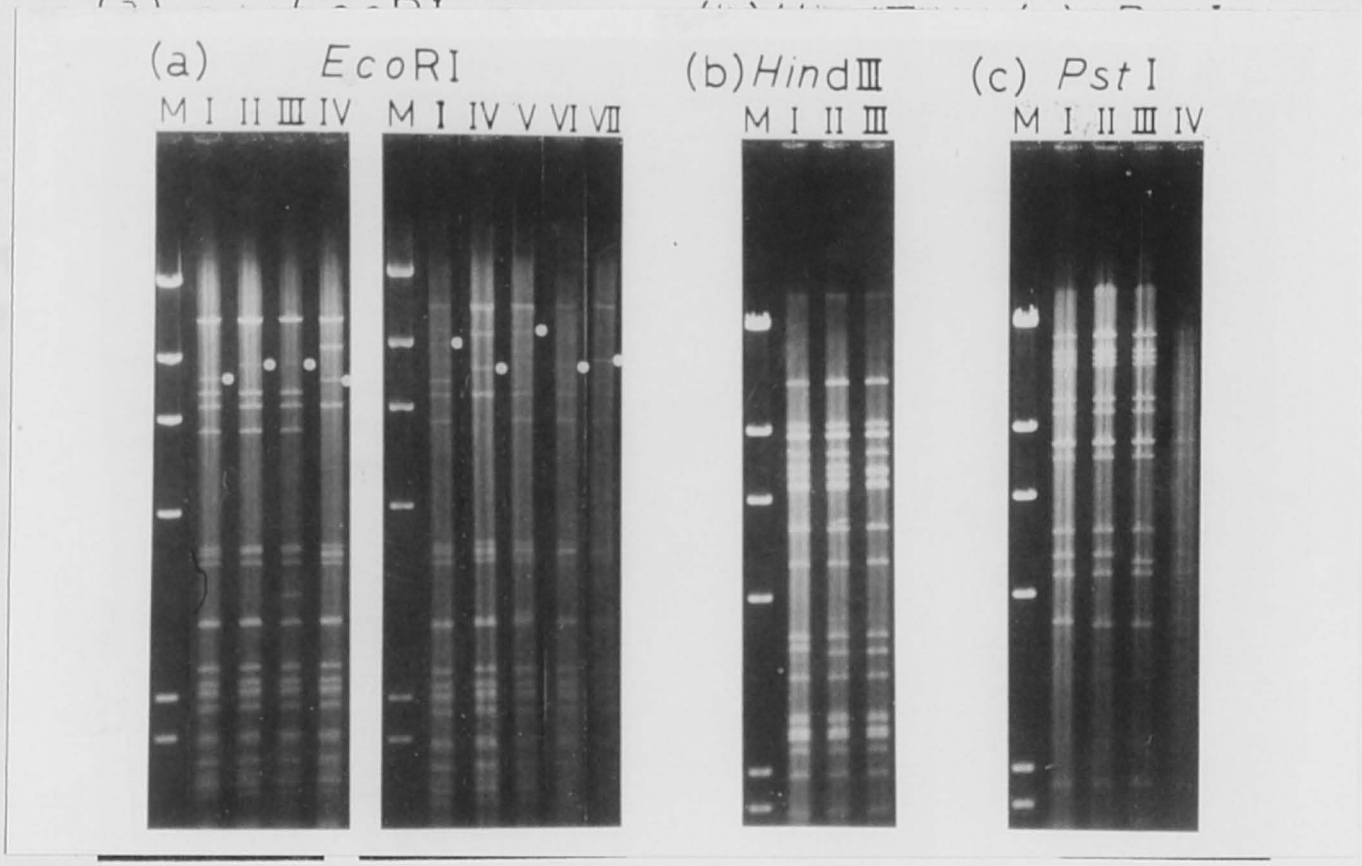


Fig. 2. EcoRI, HindIII and PstI restriction fragment patterns of ctDNA found in four A-genome diploid species. In EcoRI restriction fragment patterns, rDNA fragments (marked with the circle) which are contamination of nuclear DNA to ctDNA sample are found.

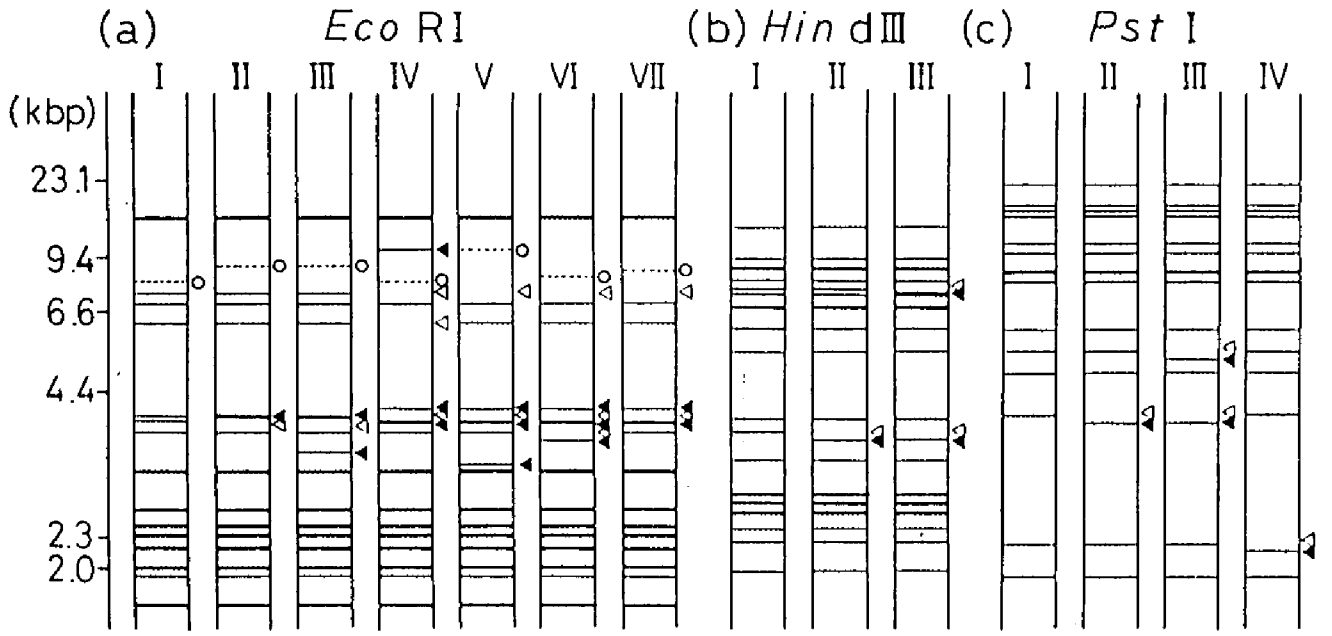


Fig. 3. Schematic representation of seven EcoRI (a), three HindIII (b) and four PstI (c) restriction fragment patterns of ctDNA found among 66 accessions of four A-genome diploid species.
◁ and ◀ : Missing and different fragment, respectively, as compared with the fragments of type I pattern. ○: rDNA fragment.

Table 5. Fragment constitutions of the restriction patterns and molecular sizes of the individual fragments identified in EcoRI, HindIII and PstI digests of ctDNA in A-genome diploid species

EcoRI								
Fragment		Type						
No.	kbp	I	II	III	IV	V	VI	VII
E1	12.6	++	++	++	++	++	++	++
--	9.8	-	-	-	+	-	-	-
E2	7.4	+	+	+	-	-	-	-
E3	6.8	+	+	+	+	+	+	+
E4	6.1	+	+	+	-	+	+	+
--	3.9	-	-	-	+	+	+	+
E5	3.8	+	+	+	-	-	-	-
--	3.8	-	+	+	-	-	-	-
E6	3.7	+	-	-	-	-	-	-
--	3.7	-	-	-	+	+	+	+
--	3.7	-	-	-	+	+	+	+
E7	3.6	+	+	+	+	+	-	+
--	3.5	-	-	-	-	-	+	-
--	3.1	-	-	+	-	-	-	-
--	3.0	-	-	-	-	+	-	-
E8	2.9	+	+	+	+	+	+	+
E9	2.9	+	+	+	+	+	+	+
E10	2.9	+	+	+	+	+	+	+
E11	2.5	++	++	++	++	++	++	++
E12	2.4	+	+	+	+	+	+	+
E13	2.4	+	+	+	+	+	+	+
E14	2.3	++	++	++	++	++	++	++
E15	2.3	+	+	+	+	+	+	+
E16	2.2	+	+	+	+	+	+	+
E17	2.2	+	+	+	+	+	+	+
E18	2.0	+	+	+	+	+	+	+
E19	2.0	+	+	+	+	+	+	+
E20	2.0	+	+	+	+	+	+	+
E21	1.8	+	+	+	+	+	+	+
E22	1.8	+	+	+	+	+	+	+

+ and ++: Single and double copy.
-: No fragment present.
Fragment designation follows to Hiratsuka et al. (1989).

Table 5. (continued)

HindIII					PstI				
Fragment		Type			Fragment		Type		
No.	kbp	I	II	III	No.	kbp	I	II	III	IV
H1	12.9	+	+	+	P1	19.2	+	+	+	+
H2	9.6	+	+	+	P2	16.2	+	+	+	+
H3	9.0	++	++	++	P3	15.2	+	+	+	+
H4	8.2	+	+	+	P4	14.4	+	+	+	+
H5	7.7	+	+	-	P5	10.9	+	+	+	+
--	7.5	-	-	+	P6	10.1	+	+	+	+
H6	7.4	+	+	+	P7	8.4	++	++	++	++
H7	7.1	+	+	+	P8	7.8	+	+	+	+
H8	6.9	+	+	+	P9	5.5	+	+	+	+
H9	5.8	+	+	+	P10	5.1	+	+	-	+
H10	5.0	+	+	+	--	4.9	-	-	+	-
H11	3.8	+	+	+	P11	4.7	+	+	+	+
H12	3.6	+	-	-	P12	3.9	+	-	-	+
--	3.5	-	+	+	--	3.8	-	-	+	-
H13	3.2	+	+	+	P13	2.2	+	+	+	-
H14	2.8	+	+	+	--	2.1	-	-	-	+
H15	2.8	+	+	+	P14	1.8	+	+	+	+
H16	2.7	++	++	++						
H17	2.6	++	++	++						
H18	2.5	+	+	+						
H19	2.3	+	+	+						
H20	2.0	+	+	+						

Table 6. Classification of 66 accessions of four *Oryza* species, based on their ctDNA restriction fragment patterns

Taxon	Cultivar	Rest. frag. pattern		
		*Eco*RI	*Hin*dIII	*Pst*I
O. sativa Japonica	Nipponbare	I	I	I
"	Norin No. 22	I	I	I
"	Taichung No. 65	I	I	I
"	Toride No. 1	I	I	I
"	Homarenishiki	I	I	I
"	Aichiasahi	I	I	I
"	Yamabiko	I	I	I
"	Akebono	I	I	I
"	Iwaimochi	I	I	I
"	501	I	I	I
"	563	I	I	I
O. sativa Javanica	242	I	I	I
"	532	I	I	I
"	647	I	I	I
"	(C5810) Taichung No. 65	I	I	I
"	(C5811) Taichung No. 65	I	I	I
O. sativa Indica	101	II	II	II
"	104	II	II	II
"	108	II	II	II
"	115	II	II	II
"	144	II	II	II
"	414	II	II	II
"	415	II	II	II
"	419	I	I	I
"	435	II	II	II
"	437	II	II	II
"	444	I	I	I
"	(868) Taichung No. 65	II	II	II
"	1034	I	I	I
"	C5444	II	II	II
"	C8005	III	III	III
"	IR36	II	II	II
"	Twx1	II	II	II
O. glaberrima	(W025) Taichung No. 65	IV	I	I
"	W401	IV	I	I
"	W438	IV	I	I
"	(W440) Fujiminori	IV	I	I
"	W446	IV	I	I
"	W492	IV	I	I
"	W528	IV	I	I
"	gl	IV	I	I

Table 6. (continued)

Taxon	Accession	Rest. frag. pattern		
		EcoRI	HindIII	PstI
O. perennis Asian	(W107) Taichung No. 65	II	II	II
" "	W108	II	II	II
" "	W120	I	I	I
" "	W149	II	II	II
" "	W593	I	II	II
" "	W630	I	I	I
" "	W1943	II	II	II
" "	W1945	I	I	I
" "	W2028	II	II	II
" American	W1167	II	II	II
" "	W1169	VI	I	IV
" "	W1185	V	I	I
" "	W1186	V	I	I
" "	W1192	VI	I	I
" African	W1414	V	I	I
" "	W1460	V	I	I
" "	W1504	V	I	I
" "	W1540	V	I	I
" "	W1608	V	I	I
" Oceanian	W1299	VII	I	I
" "	W1633	VII	I	I
O. breviligulata	W607	IV	I	I
"	W653	IV	I	I
"	W720	IV	I	I
"	W1152	IV	I	I

Table 7. Classification of the chloroplast genome types based on the EcoRI, HindIII and PstI restriction fragment patterns, and number of the accessions of four Oryza species having the different genome types

	Chloroplast genome type									Total
	1	2	3	4	5	6	7	8	9	
EcoRI frag. pattern	I	I	II	III	IV	V	VI	VI	VII	
HindIII frag. pattern	I	II	II	III	I	I	I	I	I	
PstI frag. pattern	I	II	II	III	I	I	I	IV	I	
O. sativa Japonica	11	--	--	--	--	--	--	--	--	11
" Javanica	5	--	--	--	--	--	--	--	--	5
" Indica	3	--	13	1	--	--	--	--	--	17
O. glaberrima	--	--	--	--	8	--	--	--	--	8
O. perennis Asian	3	1	5	--	--	--	--	--	--	9
" American	--	--	1	--	--	2	1	1	--	5
" African	--	--	--	--	--	5	--	--	--	5
" Oceanian	--	--	--	--	--	--	--	--	2	2
O. breviligulata	--	--	--	--	4	--	--	--	--	4
Total	22	1	19	1	12	7	1	1	2	66
%	33.3	1.5	28.8	1.5	18.2	10.6	1.5	1.5	3.0	99.9

--: No accession was detected.

restriction endonuclease analysis using the following eight endonucleases; BamHI, BglII, EcoRV, PvuII, SalI, SmaI, XbaI and XhoI.

Six of these, i.e., BamHI, EcoRV, PvuII, SalI, SmaI and XhoI, gave the same restriction fragment pattern (type I) for all 19 accessions analyzed. Two other endonucleases, BglII and XbaI, gave three (type I, II and III) and two (type I and II) different patterns, respectively. Fig. 4 is the schematic representation of the fragment patterns produced by the eight additional restriction endonucleases. The fragment constitution and the estimated molecular sizes of individual fragments, including their copy number, of each pattern are given in Table 8.

Table 9 summarizes the ctDNA restriction fragment patterns (including EcoRI, HindIII and PstI fragment patterns) of the 19 accessions of the two cultivated species. According to the previous analysis using EcoRI, HindIII and PstI, their chloroplast genomes were classified into three types, type 1, 3 and 5 (Table 7). All accessions of O. sativa ecosp. Japonica and Javanica, and a single accession of ecosp. Indica had the same chloroplast genome. Four of the five accessions of ecosp. Indica were of another type, being distingished from the former by BglII, EcoRI, HindIII, PstI and XbaI fragment patterns. The third type was found in all accessions of O. glaberrima. This type was differentiated from the first one by BglII and EcoRI fragment patterns, and from the second one by BglII, EcoRI, HindIII, PstI and XbaI fragment patterns.

The present results fully confirm the previous chloroplast genome classification, revealing further differences among the three genomes, with no difference disclosed among the accessions previously assigned for the same chloroplast genome types.

Molecular differences between type 1, 3, 4 and 5 chloroplast genomes revealed by Southern hybridization

In the previous study, type 1 and 5 chloroplast genomes are distinguished by BglII and EcoRI restriction fragment patterns. Based on the sizes of the different ctDNA fragments of both types, two different patterns are assumed to have been caused by independent base substitutions, resulting in restriction site gain or site loss.

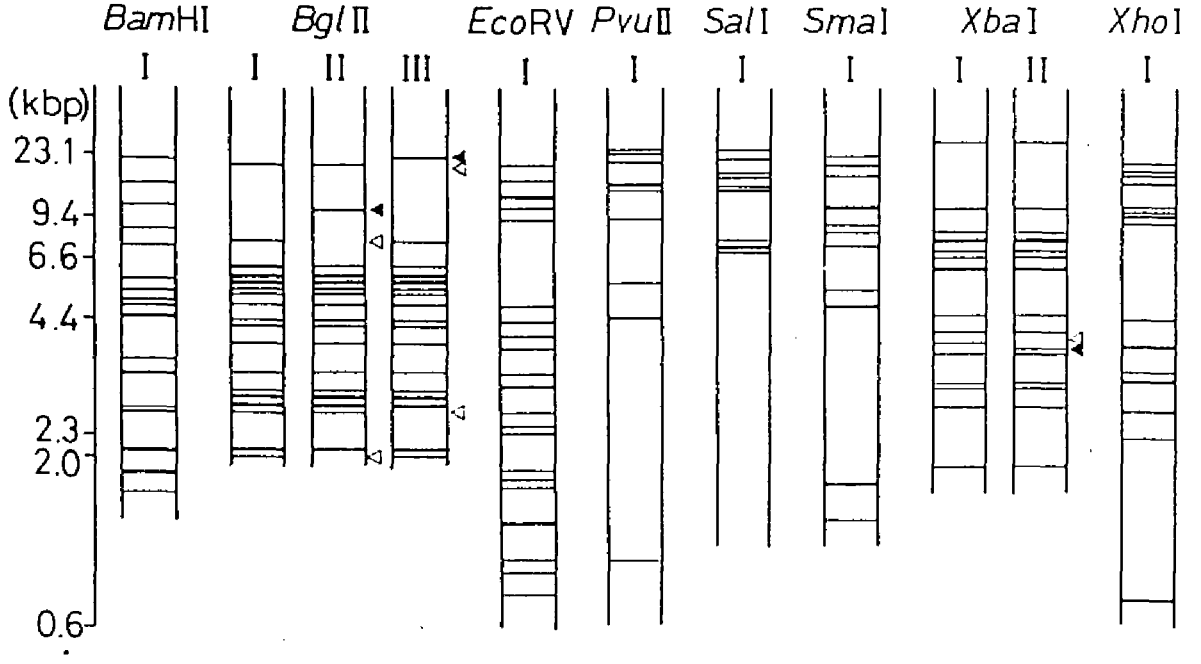


Fig. 4. Schematic representation of BamHI, BglII, EcoRV, PvuII, SalI, SmaI, XbaI and XhoI restriction fragment patterns of ctDNA found among 19 accessions of two cultivated species.
◁ and ◀: Missing and different fragment, respectively, as compared with the fragments of type I pattern.

Table 8. Fragment constitutions of the restriction fragment patterns obtained with eight endonuclease digests and the estimated molecular sizes of all fragments found among 19 cultivars of two cultivated species

BamHI			BglII				EcoRV		
Fragment		Type	Fragment		Type		Fragment		Type
No.	kbp	I	No.	kbp	I	II III	No.	kbp	I
B1	19.2	+	--	23.0	-	- +	EV1	19.5	+
B2	12.9	+	Bg1	21.6	+	+ -	EV2	15.6	+
B3	9.0	+	--	9.9	-	+ -	EV3	12.1	++
B4	8.8	+	Bg2	7.5	+	- +	EV4	11.6	+
B5	7.1	+	Bg3	5.9	+	+ +	EV5	9.9	+
B6	5.5	+	Bg4	5.4	++	++ ++	EV6	4.4	+
B7	5.0	+	Bg5	5.2	++	++ ++	EV7	4.2	+
B8	4.9	+	Bg6	5.0	+	+ +	EV8	3.7	+
B9	4.8	+	Bg7	4.8	+	+ +	EV9	3.5	+
B10	4.4	+	Bg8	4.5	+	+ +	EV10	3.0	+
B11	4.4	+	Bg9	4.2	+	+ +	EV11	2.9	++
B12	3.4	+	Bg10	4.0	+	+ +	EV12	2.5	+
B13	3.1	+	Bg11	3.6	+	+ +	EV13	2.3	+
B14	3.1	+	Bg12	3.0	+	+ +	EV14	2.2	+
B15	2.6	+	Bg13	2.8	+	+ +	EV15	1.9	+
B16	2.5	+	Bg14	2.8	++	++ ++	EV16	1.8	+
B17	2.0	+	Bg15	2.6	++	++ ++	EV17	1.7	+
B18	2.0	+	Bg16	2.5	+	+ -	EV18	1.4	++
B19	1.7	+	Bg17	2.0	++	++ ++	EV19	1.1	+
B20	1.7	+	Bg18	2.0	+	- +	EV20	1.0	+
B21	1.6	+					EV21	0.8	+

+ and ++: Single and double copy, respectively.

Table 8. (continued)

PvuII			SalI			SmaI		
Fragment		Type	Fragment		Type	Fragment		Type
No.	kbp	I	No.	kbp	I	No.	kbp	I
Pv1	26.2	+	S1	26.9	+	Sm1	24.4	+
Pv2	23.4	+	S2	22.2	+	Sm2	19.5	+
Pv3	19.7	+	S3	16.2	+	Sm3	17.2	+
Pv4	14.3	++	S4	15.8	+	Sm4	11.7	++
Pv5	14.0	+	S5	14.5	+	Sm5	9.3	+
Pv6	9.6	+	S6	14.1	+	Sm6	8.6	+
Pv7	5.2	+	S7	7.7	+	Sm7	7.5	+
Pv8	4.1	++	S8	7.4	++	Sm8	5.2	+
Pv9	1.1	+	S9	7.0	+	Sm9	4.5	++
						Sm10	1.8	++
						Sm11	1.5	+

XbaI				XhoI		
Fragment		Type		Fragment		Type
No.	kbp	I	II	No.	kbp	I
Xb1	28.3	+	+	X1	19.0	+
Xb2	11.0	+	+	X2	17.6	+
Xb3	7.9	+	+	X3	16.9	+
Xb4	7.4	++	++	X4	15.7	+
Xb5	6.9	+	+	X5	11.9	+
Xb6	6.6	+	+	X6	11.1	+
Xb7	5.9	++	++	X7	10.3	+
Xb8	4.2	+	+	X8	9.3	+
Xb9	3.8	+	+	X9	4.0	+
Xb10	3.6	+	-	X10	3.5	++
--	3.5	-	+	X11	3.1	+
Xb11	3.4	+	+	X12	3.0	++
Xb12	2.9	+	+	X13	2.5	++
Xb13	2.9	+	+	X14	2.2	+
Xb14	2.6	+	+	X15	0.7	++
Xb15	2.0	+	+			

Table 9. Classification of 19 cultivars of two cultivated species based on their ctDNA restriction fragment patterns

Ct genome type[1]	Rest. frag pattern											No. cultivars[2]
	Bam HI	Bgl II	Eco RV	Pvu II	Sal I	Sma I	Xba I	Xho I	Eco RI	Hin dIII	Pst I	
1	I	I	I	I	I	I	I	I	I	I	I	J(5/5), Jv(4/4), I(1/5)
3	I	II	I	I	I	I	II	I	II	II	II	I(4/5)
5	I	III	I	I	I	I	I	I	IV	I	I	G(5/5)

1) Previously designed in Table 7.
2) J, Jv and I: O. sativa ecosp. Japonica, Javanica and Indica, respectively. G: O. glaberrima.
( ): Number of cultivars having respective chloroplast genome / total number of cultivars studied.

Type 1 and 3 chloroplast genomes showed differences in the BglII, EcoRI, HindIII, PstI and XbaI restriction fragment patterns. To determine the nature of their ctDNA differences, ctDNAs of O. sativa ecosp. Japonica cv. Nipponbare and ecosp. Indica cv. IR36 were selected as the representative of the type 1 and 3 chloroplast genomes, respectively. EcoRI, BglII, HindIII, PstI and XbaI digests of type 1 and HindIII, PstI and XbaI digests of type 3 were Southern blotted and hybridization was performed using the cloned P12 fragment (3.9 kbp) from O. sativa ecosp. Japonica cv. Nipponbare as a probe. The results are shown in Fig. 5. The P12 fragment hybridized to the Bg1, E4, H12, P12 and Xb10 fragments of type 1, and the HindIII (3.5 kbp), PstI (3.8 kbp) and XbaI (3.5 kbp) fragments of type 3. All three fragments of type 3 were 0.1 kbp smaller than the corresponding fragments of type 1. This result indicates the fragment pattern differences found in the HindIII, PstI and XbaI digests were caused by a common 0.1 kbp length mutation.

Similarly, the HindIII fragment (H5) and the PstI fragment (P10) differences between type 1 and 4 were also determined to be caused by a common 0.2 kbp deletion in type 4. Furthermore, restriction site mapping of the P10 fragments of type 1 and 4 chloroplast genomes revealed one EcoRI site loss with this 0.2 kbp deletion in type 4 chloroplast genome (Fig. 6). Consequently, this deletion caused the EcoRI fragment differences between type 1 (2.9 + 0.4 kbp) and type 4 (3.1 kbp). Four other fragment changes were described by Dally and Second (1990). Table 10 summarizes the nature of all mutations found among four chloroplast genome types. Based on the number of the mutations detected between every pair of four chloroplast genome types (Table 11), cluster analysis using a UPGMA method (Sneath and Sokal 1973) was carried out. Fig. 7 shows the phylogenetic relationships between chloroplast genomes of ten cultivars.

Nucleotide sequence analysis on ctDNA from type 1, 3 and 5 chloroplast genemes

Restriction endonuclease analysis of ctDNA revealed that the main chloroplast genome types in cultivated rice species are type 1, 3 and 5. The following three cultivars were selected as the representative of each genome type: O. sativa ecosp. Japonica cv. Nipponbare, ecosp. Indica

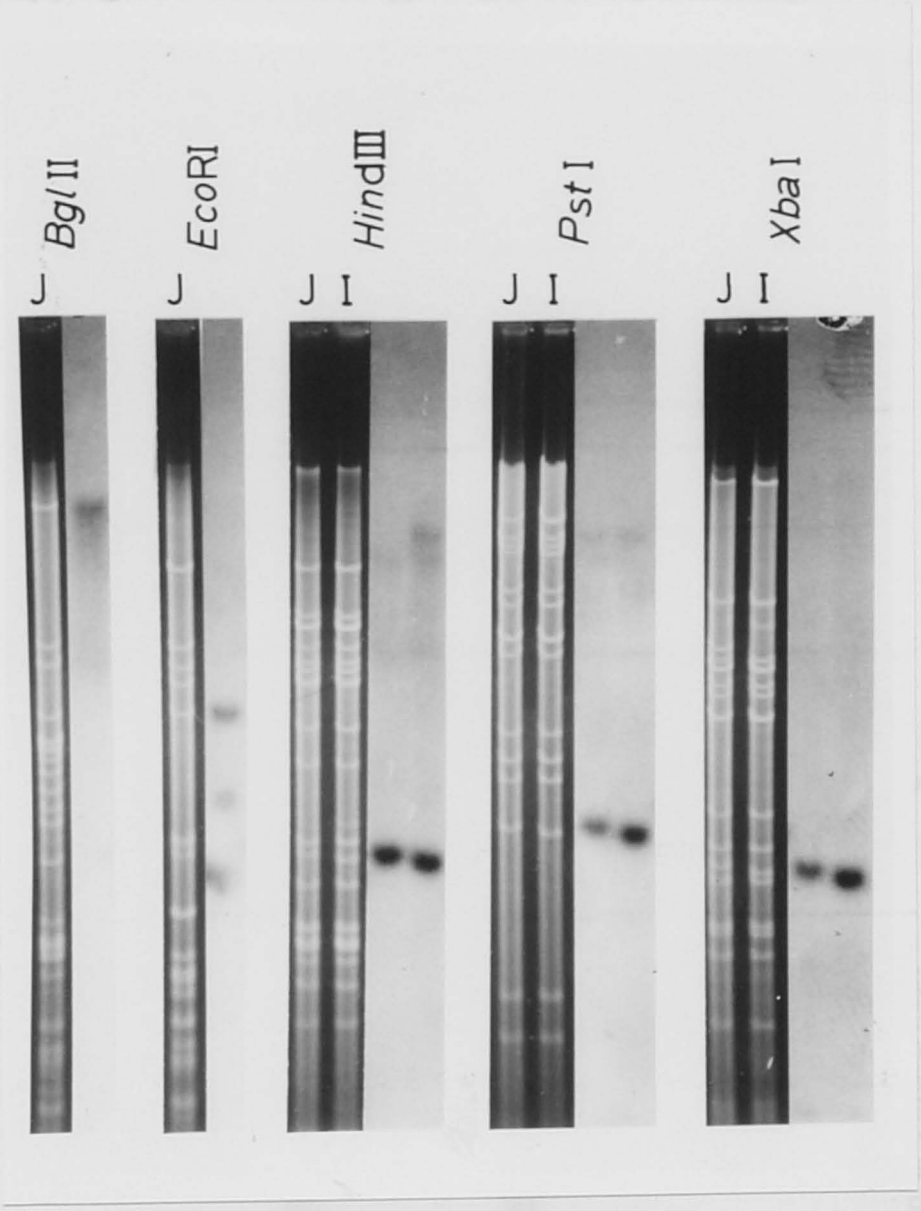


Fig. 5. Southern hybridization patterns of the P12 fragment as probe to BglII, EcoRI, HindIII, PstI and XbaI digests of O. sativa ecosp. Japonica cv. Nipponbare ctDNA (J), and to HindIII, PstI and XbaI digests of ecosp. Indica cv. IR36 (I).

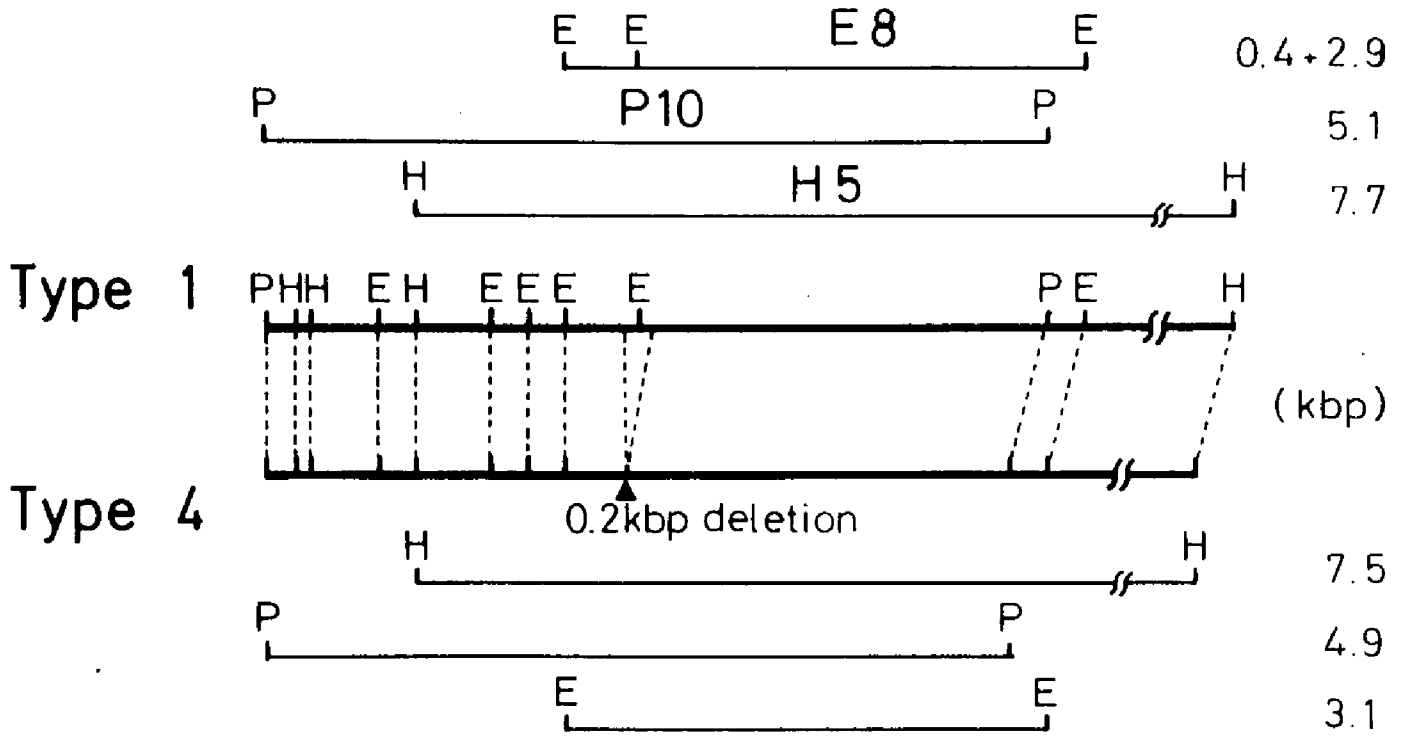


Fig. 6. Physical maps of the P10 fragments of type 1 and 4 chloroplast genomes, confirming the size of the fragments generated by EcoRI, HindIII and PstI-digestion.

Table 10. Six ctDNA mutations found in EcoRI, HindIII and PstI digests of ctDNA between type 1 and other chloroplast genomes

Mutation[1)]	Ct genomes compared	Size difference (kbp)
Insertion (0.1 kbp)	1 vs 3, 4	3.7 (E6) vs 3.8
Deletion (0.1 kbp)	1 vs 3, 4	3.6 (H12) vs 3.5 3.9 (P12) vs 3.8
Deletion (0.2 kbp)	1 vs 4	2.9 (E8) vs 3.1 7.7 (H5) vs 7.5 5.1 (P10) vs 4.9
Insertion (0.1 kbp)	1 vs 5	3.8 (E5) vs 3.9
Site gain (EcoRI)	1 vs 5	7.4 (E2) vs 3.7 + 3.7
Site loss (EcoRI)	1 vs 5	6.1 (E4) + 3.7 (E6) vs 9.8

1) Type 1 chloroplast genome is considered as the standard.

Table 11. Number of mutations found between every pair of the four chloroplast genome types

Ct genome type	1	3	4	5
1	--	2	3	3
3		--	1	5
4			--	6
5				--

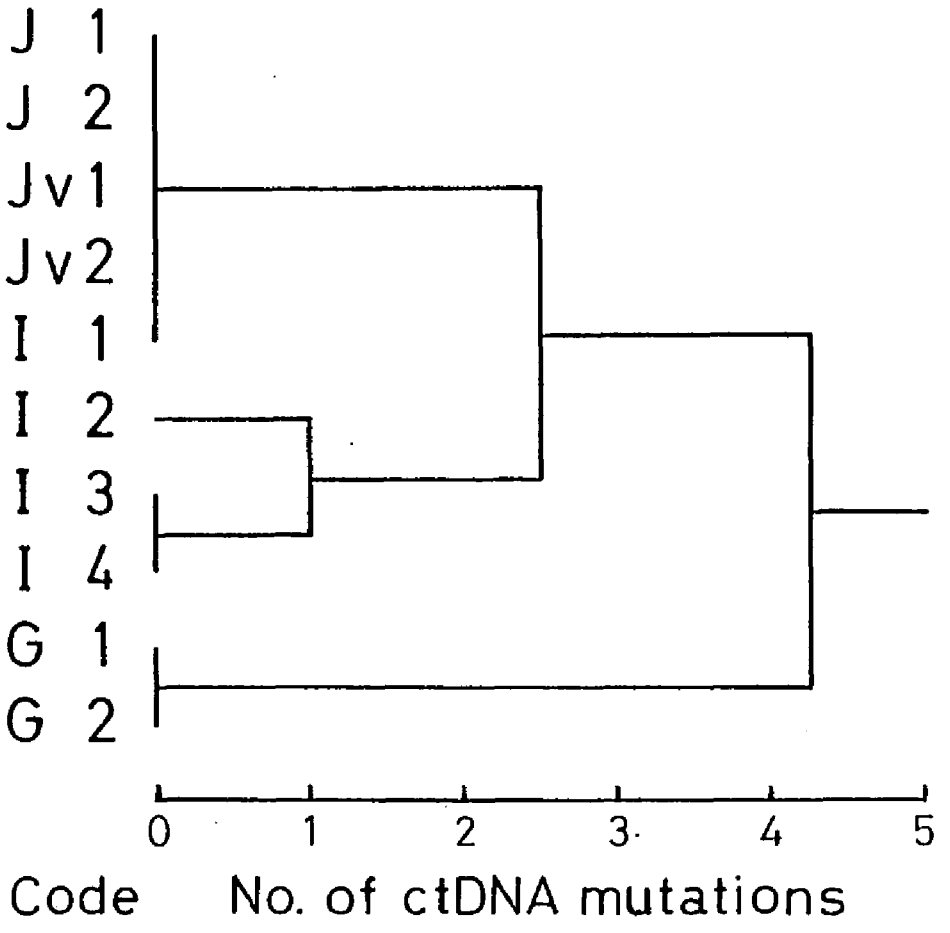


Fig. 7. Dendrogram showing genetic relationships between chloroplast genomes of ten cultivars of Oryza based on the number of ctDNA mutations.

cv. IR36, and O. glaberrima strain W401. In order to clarify the differences between them at DNA sequence level, three PstI fragments (P11, P13 and P14), which were cloned from all three cultivars, were subjected for nucleotide sequence analysis. Fig. 8. shows their location in the rice ctDNA physical map, and the strategies employed in sequencing these three fragements.

Nucleotide sequences of the both ends of the P11 fragment are shown in Fig. 9. Compared to the nucleotide sequence of maize chloroplast gene encoding the proteolipid subunit of CF_0 (atpH) (Rodermel and Bogorad 1987), one end of 391 bp is supposed to contain 97 bp of the N-terminus of the coding region of this gene, and 294 bp of its 5' flanking region. The other end of 433 bp locates in the non-coding region. In both ends, no nucleotide difference was detected among the three chloroplast genome types.

Nucleotide sequences in both ends of the P13 fragment are shown in Fig. 10. Both sequenced ends of 232 and 201 bp are located in non-coding region. The latter shows homology to about 50 bp upstream region of the maize chloroplast $tRNA^{ser}$ gene (Steinmetz et al. 1983). In both ends, no nucleotide difference was detected among the three chloroplast genome types.

Nucleotide sequences in both ends of the P14 fragment are shown in Fig. 11. Compared to the maize chloroplast gene (atpB) for the beta subunit of the photosynthetic coupling factor CF_1 (Krebbers et al. 1982), one end of 279 bp is assumed to be in the coding region of this gene. Compared to the maize chloroplast gene (rbcL) for the large subunit of ribulose 1,5-bisphosphate carboxylase (McIntosh et al. 1980), the other end of 202 bp includes N-terminal end of 169 bp of the coding region of this gene, and 33 bp of its 5' flanking region. In both ends, no nucleotide difference was detected among the three chloroplast genomes.

In total, 1738 bp of ctDNA were sequenced. Of these, 545 bp belonged to the coding regions, and 1193 bp to the non-coding regions. In both categories of the sequences, no nucleotide difference was detected among O. sativa ecosp. Japonica cv. Nipponbare, ecosp. Indica cv. IR36 and O. glaberrima strain W401.

CtDNA variation in O. sativa

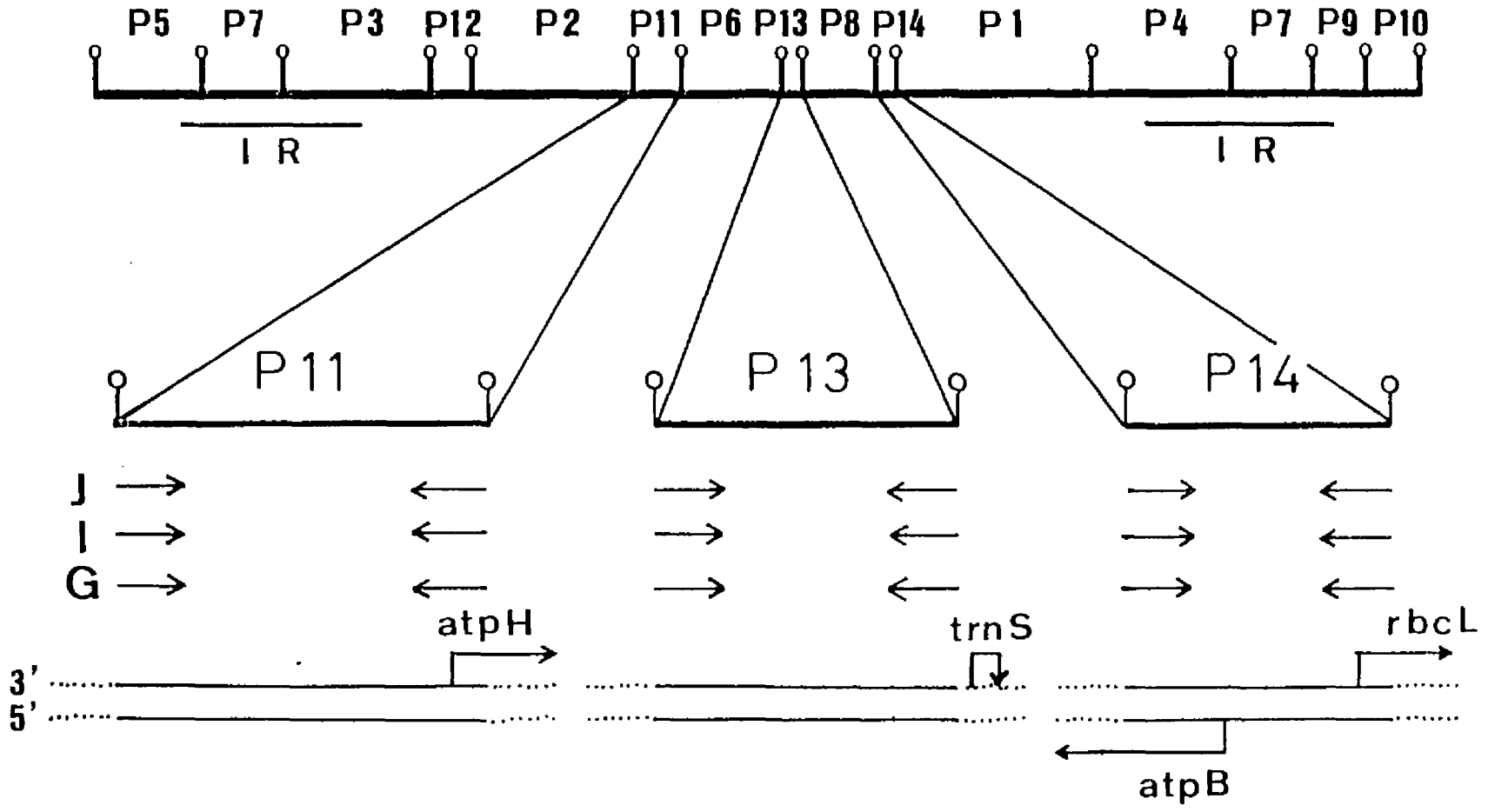


Fig. 8. Locations of three PstI fragments, P11, P13 and P14, and the strategies employed in their nucleotide sequencing.

```
        10          20          30          40          50          60
CTGCAGCAGT ACCTTGACCA ACTCCAGGCC CAATAGAAGC AAGACCTACG GCCAATCCAG

        70          80          90         100         110         120
CAGCAATAAC GGAAGCAGCA NNAATTAGTG GATTCATGAT GAGTTCCTCG TGTCAAAAAA

       130         140         150         160         170         180
AAGAAATGGT TAAGGATACA ATCAACCAAG AAATTCATAT TTCTAAGCTC TATTGGACAG

       190         200         210         220         230         240
AGTAACTAAA AAGTACAAAT TGAAACGATA ATCTGAATTC TCCGAACTGC TTCGAGATCT

       250         260         270         280         290         300
CCTTTTTAGT TTCTAATCAT TAGAGGTTTG TGTACTCATT ATTCTATTTC TCTTTCTTTC

       310         320         330         340         350         360
CAACCAACTG ATCTTTCATT CCATCCTTCT TTCTTTCCTC TTCGATATCC TTGAGTTTCT

       370         380         390         400
ATTTTTTCCC CTATCATCTA ATTCATAATA A
```

```
        10          20          30          40          50          60
CTGCAGTGCG TATGAAATGA ACGAAGGGAG AAATTTAGCA ACACTTTTCC CACAGGATCT

        70          80          90         100         110         120
CTTGCAGGAA GAGGGTAATC TCCAACTTCG ACTTGTCAAT TTTATTTCTC ATGAAAATAG

       130         140         150         160         170         180
CAAGTTAACT CAAAGAATTT ATCATACGAA TAGTCAATTC GTTCGAACTT GCTTAGTATG

       190         200         210         220         230         240
AATTGGGAAC AAGAAGAAAA AGAGGAGGCT CGTGCTTCCC TTGTTGAGAT AAGAGCAAAT

       250         260         270         280         290         300
GGTCTGATTC GCGATTTCCT AAGAATTGGG TTAATCAAAT CCACTATTTC GTATACACGA

       310         320         330         340         350         360
AAAGGTATGA TAGCAGAAGT GCAGGACTGA TTCTCCATAA TAGGTTAGAT CGCACCAATA

       370         380         390         400         410         420
CCAACCTTTT ATTCCAAGGC GAAGATTCAA TCACTTAGCC AACATCAAGA AGCTATTGGT

       430         440
ACCTTGTTGA ATC
```

Fig. 9. Nucleotide sequences of both ends of the P11 fragment cloned from type 1, 3 and 5 chloroplast genomes. The completely identical nucleotide sequences were obtained from the three chloroplast genomes.

```
         10          20          30          40          50
CTGCAGAAAA ATGAAGCATA GATAGACCTA TATCCTTCGT CCGAATTTTC

         60          70          80          90          100
TGAAAGGTAA CTATCTCGGT TTCATATATG AAATTTCTAT AGAATCCTTG

         110         120         130         140         150
AAAAAGACTT TTTCCCCATA AGCAAGAAAA AAGAACTTAC TATCTTTGGG

         160         170         180         190         200
ATCTGAGACT ACACCGCTGC TTAATCCCTT AGTGGATCGG CTCTATTACA

         210         220         230         240
TAAGCGGATT CCTAAATTTT GCCCCATATC AT
```

```
         10          20          30          40          50
CTGCAGTACA GGTACAACCA CAACCGCGCG AGAGTTCCAT TGTTCTATTA

         60          70          80          90          100
GATAGAAAAA TTCCTTTTCA TCTAAGTGGA CGGGTCCAGG ATTTTTTTAC

         110         120         130         140         150
TAGGAATTCC GCTCCCTCGA AAAGTTTTAG TTTGGGTTTT CCCAAACCAA

         160         170         180         190         200
AGAAAAAGAG AATGGAAGAA TTCTTCTTGT TCGATAAAAA AGGAACCCTA

         210
G
```

Fig. 10. Nucleotide sequences of both ends of the P13 fragment cloned from type 1, 3 and 5 chloroplast genomes. The identical nucleotide sequences were obtained from the three chloroplast genomes.

```
        10          20          30          40          50
CTGCAGAGGG CATTCTCCCT AATAAGGCAG ATACCTCCGA TCCTGCTTGA

        60          70          80          90         100
ACAAAACGAA AGATATTATC GATGAATAGA AGCACGTCTT GCTTATTAAC

       110         120         130         140         150
ATCTCGGAAA TATTCTGCCA TAGTTAGGGC AGTCAAACCA ACTCTCATAC

       160         170         180         190         200
GAGCTCCTGG CGGTTCATTC ATTTGGCCAT AGACTAGAGC TACCTTTGAT

       210         220         230         240         250
TCCTCAAGAT TTTTTTCATT AATTACTCCA GATTCCTTCA TTTCCATATA

       260         270         280
AAGATCATTT CCTTCACGAG TCCGTTCCC
```

```
        10          20          30          40          50
CTGCAGCCCC TGCTTCTTCG GGCGGAACCC CCGGCTGAGG AGTTACTCGG

        60          70          80          90         100
AATGCTGCCA AGATATCAGT GTCCTTGGTT TCGTACTCCG GGGTGTAGTA

       110         120         130         140         150
AGTCAATTTA TAATCCTTAA CACCAGCTTT AAATCCAACA CTTGCTTTAG

       160         170         180         190         200
TTTCTGTTTG TGGTGACATA CGTCCCTCCC TACAACTCAT GAATTAAGAA

       210
TT
```

Fig. 11. Nucleotide sequences of both ends of the P14 fragment cloned from type 1, 3 and 5 chloroplast genomes. The identical nucleotide sequences were obtained from the three chloroplast genomes.

Seventy five cultivars (No. 2-73, 113-115), of which isozyme constitutions were already analyzed by Glaszmann(1985), were selected from the materials given in Table 3. Restriction fragment patterns of ctDNA from 68 out of 75 cultivars could be analyzed with all six restriction endonucleases, EcoRI, HindIII, PstI, PvuII, SmaI and XhoI. Two of them, PvuII and XhoI, gave the same restriction fragment pattern (type I) for all 68 cultivars. Four other endonucleases, i.e., EcoRI, HindIII, PstI and SmaI, gave four (type I, II, VIII and IX), three (type I, II and IV), two (type I and II) and two (type I and II) different patterns, respectively (Figs. 12 and 13). The fragment constitution of each pattern and molecular sizes of individual fragments including their copy number are given in Table 12. Table 13 shows the ctDNA restriction fragment patterns of 68 cultivars and seven cultivars, for which only incomplete data are available. Based on the restriction fragment patterns, the chloroplast genomes of 68 cultivars could be classified into five types (Table 14).

The fragment differences detected between type 1 and other chloroplast genome types are given in Table 15. In order to identify the nature and the location of the mutation on the PstI physical map, Southern hybridization was carried out. Using the P12 fragment as a probe, a 0.1 kbp difference was detected in both H12 and P12 fragments between type 1 and type 3 chloroplast genomes, indicating that they share a common 0.1 kbp length mutation.

To clarify the nature of other changes, the restriction fragments of the type 1 chloroplast genome, which show a change in other chloroplast genome(s), were recovered from the agarose gel and used as probes. Firstly, they were hybridized to the ctDNAs of other chloroplast genome types digested with the same endonucleases (Table 16a). From the results, the changes observed in the E4, E6 and E7 fragments were identified to be caused by a 0.3 kbp insertion in type 12 chloroplast genome, a 0.1 kbp insertion in types 3 and 10, and a 0.1 kbp deletion in types 11 and 12, respectively. Changes in the H2 and Sm2 fragments were identified to be caused by the addition of new restriction site (site gain) in type 10 chloroplast genome.

Secondly, the fragments showing differences between type 1 and other chloroplast genomes were hybridized with the PstI digest of ctDNA from the type 1 genome to locate them on

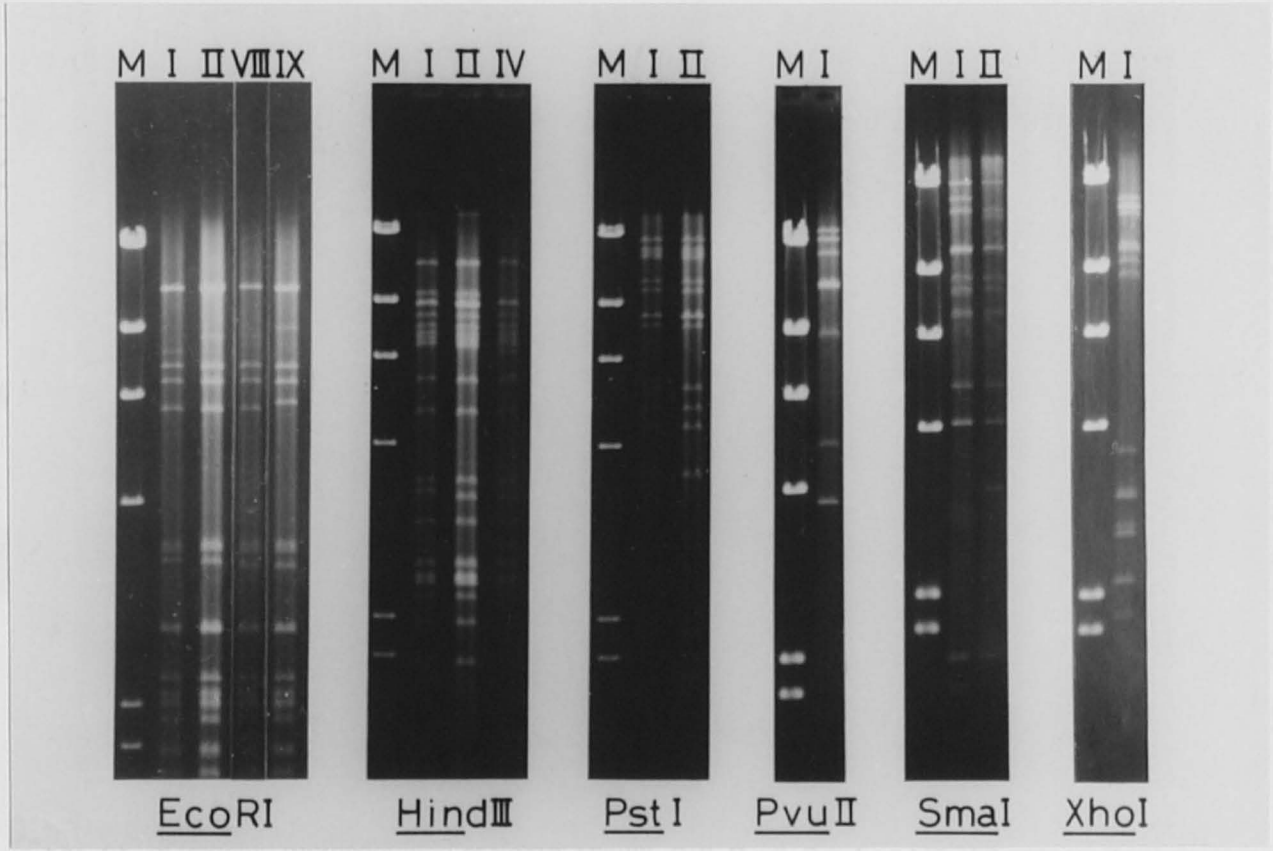


Fig. 12. EcoRI, HindIII, PstI, PvuII, SmaI and XhoI restriction fragment patterns of ctDNA observed among 68 Asian cultivars of O. sativa.

M: Lambda DNA digested with HindIII as molecular markers.

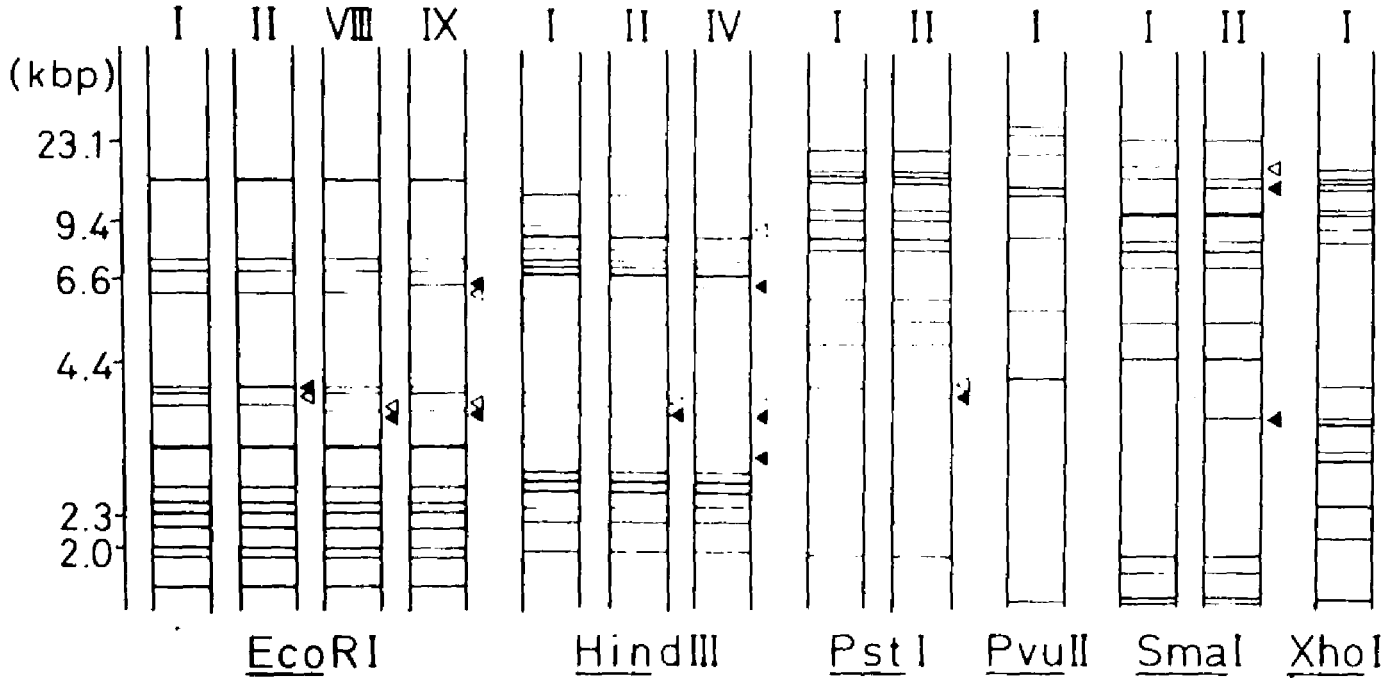


Fig. 13. Schematic representation of EcoRI, HindIII, PstI, PvuII, SmaI and XhoI restriction fragment patterns of ctDNA observed among 68 Asian cultivars of O. sativa.

◁ and ◀: Missing and different fragment, respectively, as compared with the corresponding fragments of the type I pattern.

Table 12. Fragment constitutions of the restriction patterns and molecular sizes of the individual fragments identified in six endonuclease digests of ctDNA from 68 cultivars of O. sativa

EcoRI						HindIII					PvuII		
Fragment		Type				Fragment		Type			Fragment		Type
No.	kbp	I	II	VIII	IX	No.	kbp	I	II	IV	No.	kbp	I
E1	12.6	++	++	++	++	H1	12.9	+	+	+	Pv1	29.1	+
E2	7.4	+	+	+	+	H2	9.6	+	+	-	Pv2	23.9	+
E3	6.8	+	+	+	+	H3	9.0	++	++	++	Pv3	18.6	+
--	6.4	-	-	-	+	H4	8.2	+	+	+	Pv4	13.2	++
E4	6.1	+	+	+	-	H5	7.7	+	+	+	Pv5	12.8	+
E5	3.8	+	+	+	+	H6	7.4	+	+	+	Pv6	9.1	+
--	3.8	-	+	-	-	H7	7.1	+	+	+	Pv7	5.3	+
E6	3.7	+	-	+	+	H8	6.9	+	+	+	Pv8	4.1	++
E7	3.6	+	+	-	-	--	6.6	-	-	+	Pv9	1.0	+
--	3.5	-	-	+	+	H9	5.8	+	+	+			
E8	2.9	+	+	+	+	H10	5.0	+	+	+			
E9	2.9	+	+	+	+	H11	3.8	+	+	+			
E10	2.9	+	+	+	+	H12	3.6	+	-	-			
E11	2.5	++	++	++	++	--	3.5	-	+	+			
E12	2.4	+	+	+	+	H13	3.2	+	+	+			
E13	2.4	+	+	+	+	--	3.0	-	-	+			
E14	2.3	++	++	++	++	H14	2.8	+	+	+			
E15	2.3	+	+	+	+	H15	2.8	+	+	+			
E16	2.2	+	+	+	+	H16	2.7	++	++	++			
E17	2.2	+	+	+	+	H17	2.6	++	++	++			
E18	2.0	+	+	+	+	H18	2.5	+	+	+			
E19	2.0	+	+	+	+	H19	2.3	+	+	+			
E20	2.0	+	+	+	+	H20	2.0	+	+	+			
E21	1.8	+	+	+	+								
E22	1.8	+	+	+	+								

Note) Fragment designation follows to Hiratsuka et al. (1989).
+ and ++: Single and double copy, respectively.
-: No fragment present.

Table 12. (continued)

PstI				SmaI				XhoI		
Fragment		Type		Fragment		Type		Fragment		Type
No.	kbp	I	II	No.	kbp	I	II	No.	kbp	I
P1	19.2	+	+	Sm1	23.0	+	+	X1	16.9	+
P2	16.2	+	+	Sm2	17.4	+	-	X2	15.6	+
P3	15.2	+	+	Sm3	15.0	+	+	X3	15.2	+
P4	14.4	+	+	--	14.0	-	+	X4	14.1	+
P5	10.9	+	+	Sm4	10.7	+	+	X5	10.8	+
P6	10.1	+	+	Sm5	10.7	++	++	X6	10.5	+
P7	8.4	++	++	Sm6	8.8	+	+	X7	9.7	+
P8	7.8	+	+	Sm7	8.3	+	+	X8	8.9	+
P9	5.5	+	+	Sm8	7.3	+	+	X9	4.0	+
P10	5.1	+	+	Sm9	5.2	+	+	X10	3.4	+
P11	4.7	+	+	Sm10	4.4	++	++	X11	3.3	++
P12	3.9	+	-	--	3.4	-	+	X12	3.0	+
--	3.8	-	+	Sm11	1.8	++	++	X13	2.9	++
P13	2.2	+	+	Sm12	1.6	+	+	X14	2.4	++
P14	1.8	+	+	Sm13	1.1	++	++	X15	2.1	+
				Sm14	1.0	+	+	X16	0.9	++

Table 13. Classification of 75 cultivars of O. sativa based on their ctDNA restriction fragment patternsMaterials used as the source of chloroplast and nuclear DNA

No.	Acc.No	Rest. frag. Pattern						Ct genome type
		EcoRI	HindIII	PstI	PvuII	SmaI	XhoI	
1	IR36	II	II	II	I	I	I	3
2	328	I	I	I	I	I	I	1
3	1107	I	I	I	I	I	I	1
4	1112	I	I	I	I	I	I	1
5	1254	I	I	I	I	I	I	1
6	3688	II	II	II	I	I	I	3
7	3717	I	I	I	I	I	--	(1)
8	5423	II	II	II	--	--	--	(3)
9	6046	I	I	I	I	I	I	1
10	6246	I	I	I	I	I	I	1
11	6264	II	II	II	I	I	I	3
12	6267	II	II	II	I	I	--	(3)
13	6274	I	I	I	I	I	I	1
14	6294	II	II	II	I	I	--	(3)
15	6304	I	I	I	I	--	--	(1)
16	6307	II	II	II	I	I	I	3
17	6331	II	II	II	I	I	I	3
18	6386	I	I	I	I	I	I	1
19	6422	--	I	I	I	I	I	(1)
20	6426	I	I	I	I	I	I	1
21	6538	II	II	II	I	I	I	3
22	6541	I	I	I	I	I	I	1
23	6550	II	II	II	I	I	I	3
24	7722	II	II	II	I	I	I	3
25	7755	II	II	II	I	I	I	3
26	8896	I	I	I	I	I	I	1
27	8952	II	II	II	I	I	I	3
28	8972	II	II	II	I	I	I	3
29	9177	II	II	II	I	I	I	3
30	12881	IX	I	I	I	I	I	12
31	17052	I	I	I	I	I	I	1
32	17054	I	I	I	I	I	I	1
33	23364	I	I	I	I	I	I	1
34	26276	I	I	I	I	I	I	1
35	27509	I	I	I	I	I	I	1
36	27513	I	I	I	I	I	I	1
37	27516	II	II	II	I	I	I	3
38	27536	I	I	I	I	I	I	1
39	27590	II	IV	II	I	II	I	10
40	27762	II	II	II	I	I	I	3

Table 13. (continued)

No.	Acc.No	Rest. frag. Pattern						Ct genome type
		EcoRI	HindIII	PstI	PvuII	SmaI	XhoI	
41	27798	I	I	I	I	I	I	1
42	30238	II	II	II	I	I	I	3
43	30342	I	I	I	I	I	I	1
44	31525	I	I	I	I	I	I	1
45	32292	IX	I	I	I	I	I	12
46	32300	II	II	II	I	I	I	3
47	32561	II	II	II	I	I	I	3
48	33187	I	I	I	I	I	I	1
49	33188	I	I	I	I	I	I	1
50	33192	I	I	I	I	I	I	1
51	33888	I	I	I	I	I	I	1
52	38698	I	I	I	I	I	I	1
53	40673	II	II	II	I	I	I	3
54	43369	II	II	II	I	I	I	3
55	43372	I	I	I	I	I	I	1
56	43394	I	I	I	I	I	I	1
57	43400	II	II	II	I	I	I	3
58	43530	VIII	I	I	I	I	I	11
59	43540	II	II	II	I	I	I	3
60	43675	I	I	I	I	I	I	1
61	45624	II	II	II	I	I	I	3
62	45975	II	II	II	I	I	I	3
63	47529	II	II	II	I	I	I	3
64	51064	II	II	II	I	I	I	3
65	51250	II	II	II	I	I	--	(3)
66	51300	II	II	II	I	I	I	3
67	51350	II	II	II	I	I	I	3
68	51400	II	II	II	I	I	I	3
69	55457	I	I	I	I	I	I	1
70	56036	II	II	II	I	I	I	3
71	58278	II	II	II	I	I	I	3
72	58881	I	I	I	I	I	I	1
73	58930	I	I	I	I	I	I	1
113	25901	I	I	I	I	I	I	1
114	27748	II	II	II	I	I	I	1
115	39261	I	I	I	I	I	I	1

Table 14. Five chloroplast genome types found among 68 Asian cultivars of O. sativa from the EcoRI, HindIII, PstI, PvuII, SmaI and XhoI restriction fragment patterns of their ctDNAs

Ct genome type	Restriction fragment pattern						No. of cultivars
	EcoRI	HindIII	PstI	PvuII	SmaI	XhoI	
1	I	I	I	I	I	I	34
3	II	II	II	I	I	I	30
10	II	IV	II	I	II	I	1
11	VIII	I	I	I	I	I	1
12	IX	I	I	I	I	I	2
Total							68

Table 15. Restriction fragment differences between type 1 and other chloroplast genomes in their EcoRI, HindIII, PstI and SmaI digests

Ct genomes compared	Enzyme	Fragment	Size difference (kbp)
1 vs 3	EcoRI	E6	3.7 vs 3.8
"	HindIII	H12	3.6 vs 3.5
"	PstI	P12	3.9 vs 3.8
1 vs 10	EcoRI	E6	3.7 vs 3.8
"	HindIII	H2	9.6 vs 6.6 + 3.0
"	HindIII	H12	3.6 vs 3.5
"	PstI	P12	3.9 vs 3.8
"	SmaI	Sm2	17.4 vs 14.0 + 3.4
1 vs 11	EcoRI	E7	3.6 vs 3.5
1 vs 12	EcoRI	E4	6.1 vs 6.4
"	EcoRI	E7	3.6 vs 3.5

Table 16. The fragments hybridized to the probe: (a) Restriction fragments of ctDNAs digested with the same endonuclease as probe used and (b) PstI fragments.

(a)

Probe (kbp)	Ct genome type	Enzyme	Hybridized fragment (kbp)
E4 (6.1)	12	EcoRI	6.4
E6 (3.7)	3, 10	"	3.8
E7 (3.6)	11, 12	"	3.5
H2 (9.6)	10	HindIII	6.6 + 3.0
Sm2 (17.4)	10	SmaI	14.0 + 3.4

(b)

Probe	PstI hybridized fragment
E4	P2, P12
E6	P2
E7	P1
H2	P1, P8, P14
Sm2	P5, P9, P10

Table 17. Number of mutations found between every pair of the five chloroplast genome types

Ct genome type	12	11	1	3	10
12	--	1	2	4	6
11		--	1	3	5
1			--	2	4
3				--	2
10					--

the PstI physical map (Table 16b). Based on the results, the E6 and E7 fragments were found to be located in the P2 and P1 fragment, respectively. The E4 fragment hybridized with two PstI fragments, P2 and P12, and the 0.3 kbp insertion in E4 was confirmed to be in the region represented by the P2 fragment using 0.5 % agarose gel (Fig. 14). The H2 fragment hybridized with three PstI fragments, P1, P8 and P14. Hybridization of the HindIII digests with three PstI fragments as probes revealed that the mutation in the type 10 chloroplast genome resulting in the HindIII site gain occurred in the P1 fragment (Fig. 15). The Sm2 fragment also hybridized to three PstI fragments, P5, P9 and P10. Hybridization of the SmaI digests to three PstI fragments as probes revealed that two of three (P5 and P9) gave the same hybridization patterns because they contain a common sequence of the inverted repeats (Fig. 16). Another Southern hybridization was carried out using two fragments (A and B in Fig. 16) as probes, which are free of any inverted repeat sequences. The results indicated that the SmaI site gain mutation is present in the P5 fragment.

Fig. 17 shows the physical map differences among the five chloroplast genome types, in which four length mutations and two base substitutions are located. The number of the mutations detected between every pair of five chloroplast genome types is given in Table 17, based on which a dendrogram was constructed by UPGMA method to indicate genetic relationships among the five chloroplast genomes (Fig. 18).

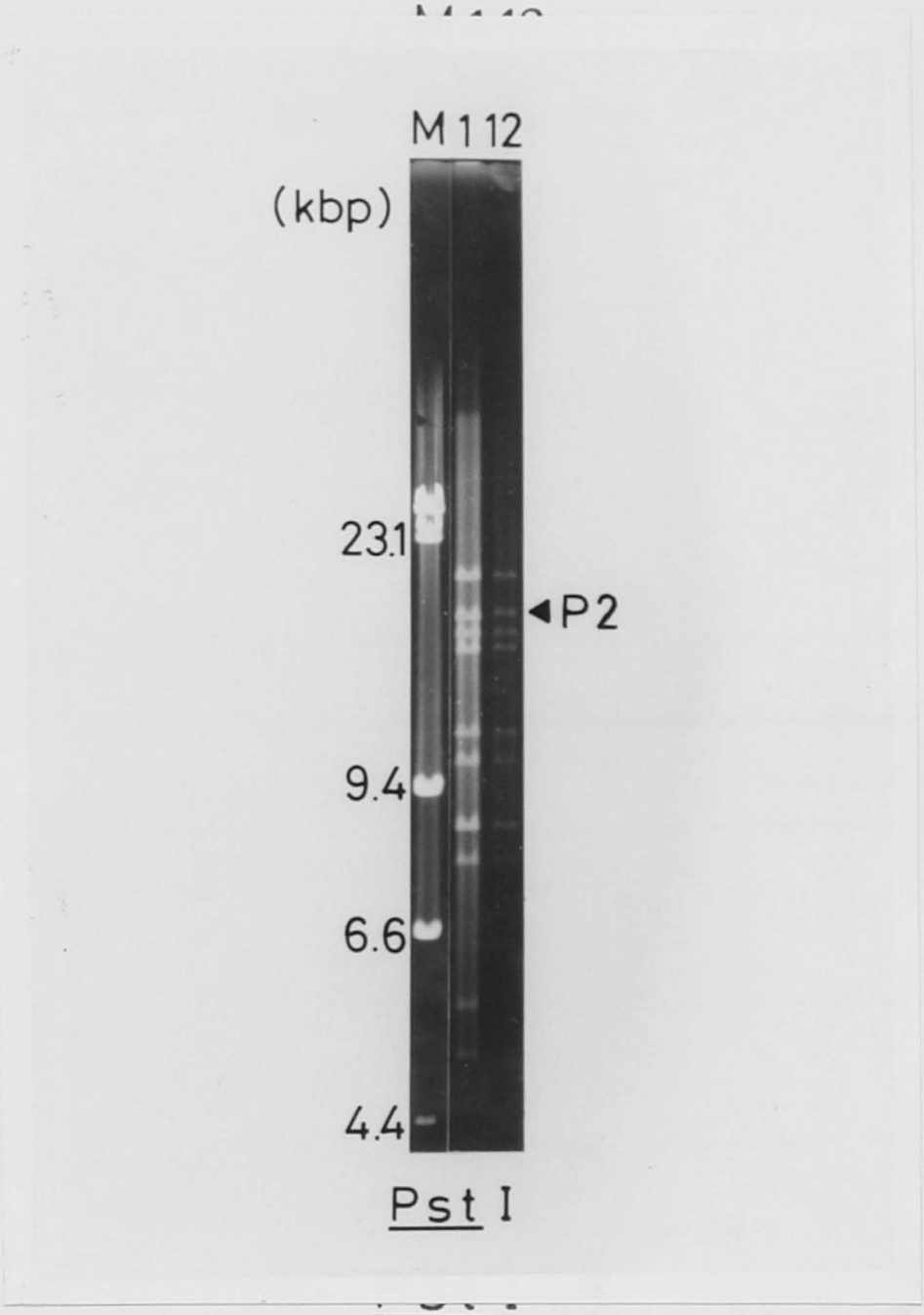


Fig. 14. PstI restriction fragment patterns of ctDNA from the type 1 and 12 chloroplast genomes using 0.5% agarose gel.

◀ indicates the different fragment between the type 1 and 12, which can not be distinguished in 0.8% agarose gel.

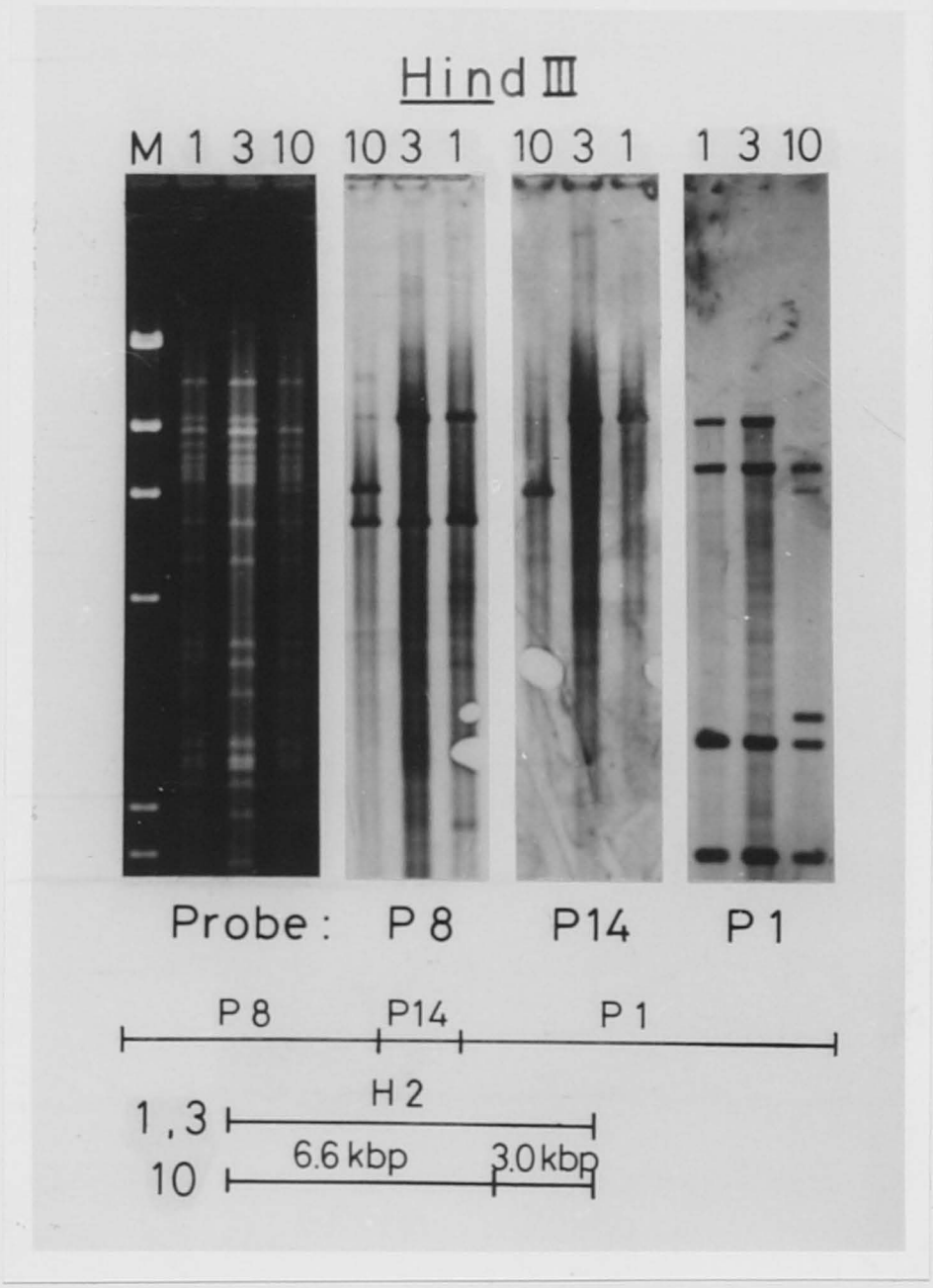


Fig. 15. Hybridization patterns of the P8, P14 and P1 fragments as probes to the HindIII fragments from the type 1, 3 and 10 chloroplast genomes, confirming the location of the site gain in the H2 fragment and the corresponding region of the PstI physical map.

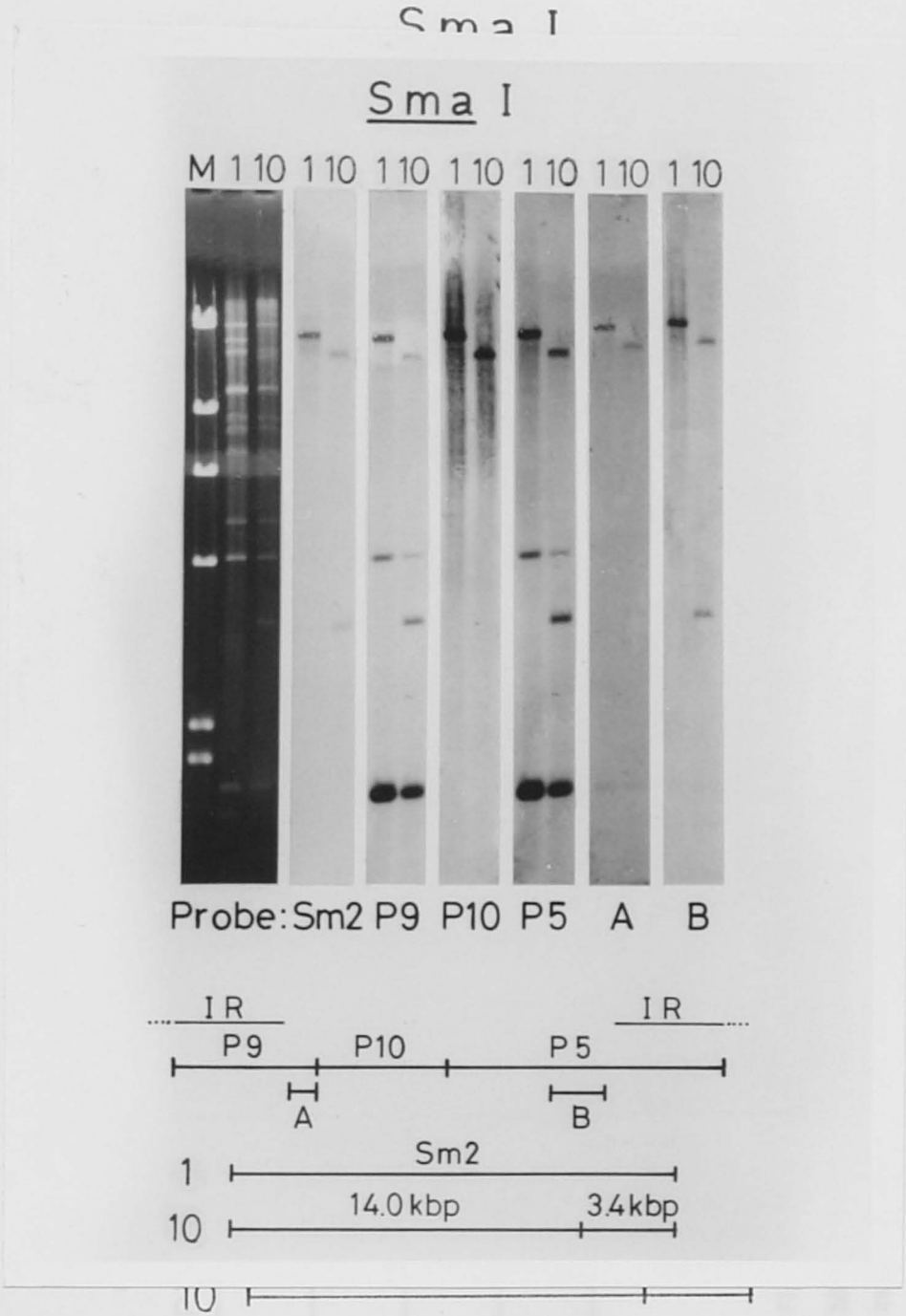


Fig. 16. Hybridization patterns of the Sm2, P9, P10, P5, A and B fragments as probes to the SmaI fragments from the type 1 and 10 chloroplast genomes, confirming the location of the site gain in the Sm2 fragment and the corresponding region of the PstI physical map. A and B are a 0.89 kbp (EcoRI-PstI) fragment generated from the P9 fragment and a 1.96 kbp (BglII-BglII) fragment from the P5 fragment, respectively. Inverted repeat sequences (IR) are shown with a solid line.

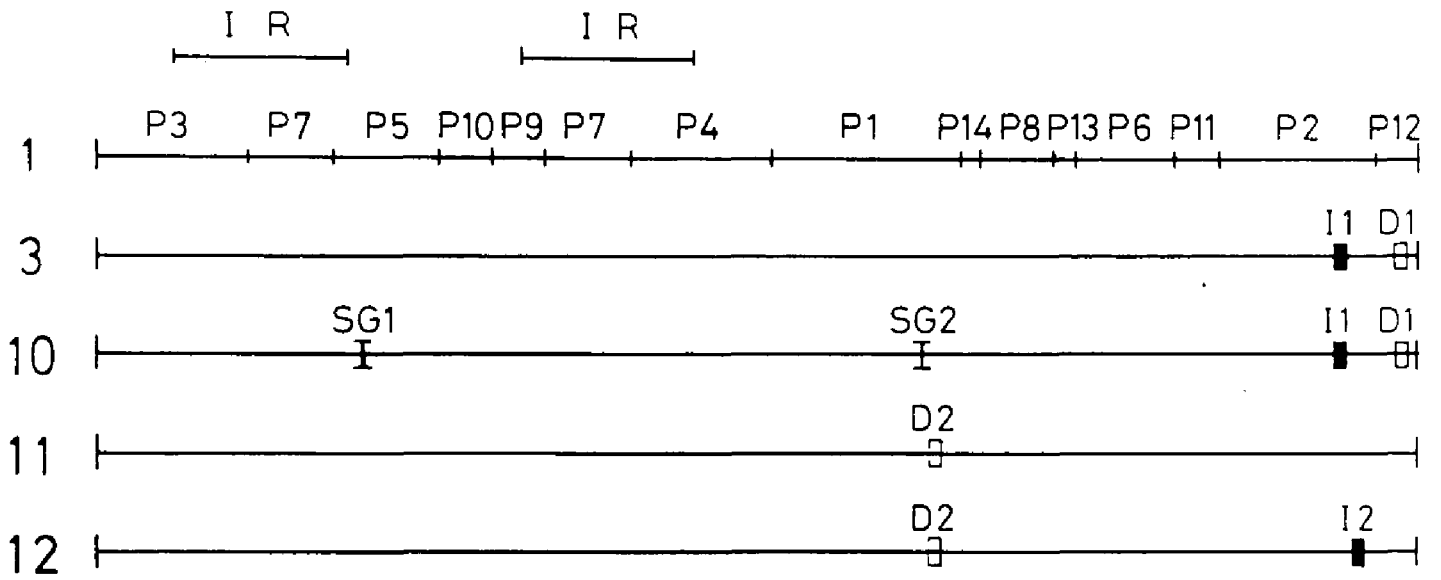


Fig. 17. Physical map differences of five chloroplast genome types found among 68 cultivars of O. sativa. On the top, PstI physical map in the type 1 chloroplast genome is shown. Mutations are indicated in the maps of the type 3, 10, 11 and 12 chloroplast genome as compared with the physical map of type 1 genome. I1, I2, D1, D2, SG1 and SG2 indicate 0.1kbp insertion, 0.3 kbp insertion, 0.1 kbp deletion, 0.1 kbp deletion, SmaI site gain and HindIII site gain, respectively.

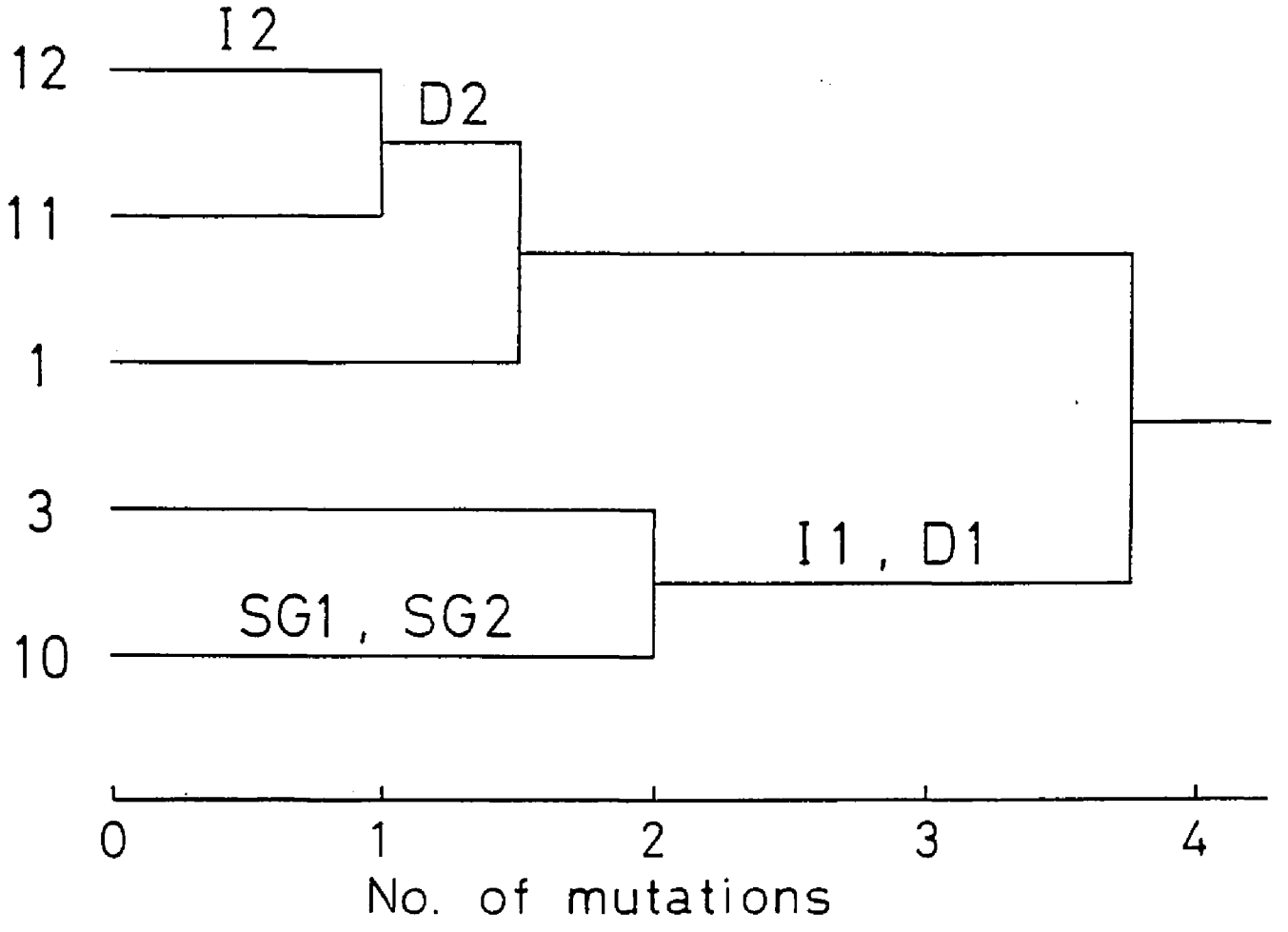


Fig. 18. Dendrogram showing the phylogenetic differentiation of five chloroplast genome types found in 68 cultivars of O. sativa. Mutations occurred in each branch are indicated. The symbols for mutations are given in Fig. 17.

B. Mitochondrial genome differentiation between O. sativa and O. glaberrima

Restriction endonuclease analysis of mtDNA

Eight cultivars of O. sativa (J1, J2, Jv1, Jv2, I1, I2, I3 and I4) and two cultivars of O. glaberrima (G1 and G2) were selected from the materials given in Table 2. Their mtDNAs were digested with five restriction endonucleases, BamHI, HindIII, PstI, PvuII and XhoI. PstI and PvuII restriction fragment patterns obtained by 1.2 % agarose gel electrophoresis are given in Fig. 19 and 20. In each restriction pattern, fragments larger than about 2 kbp were compared. However, it is difficult to identify the mutations occurred in mtDNA because of its intra- as well as intermolecular recombinations (Sederoff 1987). Alternatively, The following percentage of common fragments between two mtDNAs was used as an index of their similarity:

Percentage of common fragments = $2b / (a+a') \times 100$,

where a and a' are the numbers of total fragments in each mtDNA, and b is the number of common fragments (Nei and Li 1979).

Table 18 shows the number of the total fragments compared and the percentage of common fragments observed between ten cultivars. Based on the data given in Table 18, a dendrogram is constructed using a UPGMA method to show differentiation of the mitochondrial genome between ten cultivars (Fig. 21).

Southern hybridization analysis of mtDNA

After the restriction fragment patterns of BamHI, HindIII, PstI, PvuII and XhoI were analyzed, mtDNAs were transferred to Nylon membrane, and hybridized with four mtDNA probes, i.e., ATPA and COXII from pea (Morikami and Nakamura 1987), and RRN18&5 and RRN26 from wheat (Falconet et al. 1984, 1985). Fig. 22 shows the BamHI restriction fragment patterns of mtDNA (a) and Southern hybridization patterns of the same mtDNA with RRN18&5 probe (b). In total, 20 combinations of hybridization patterns (5 endonucleases X 4 probes) were obtained.

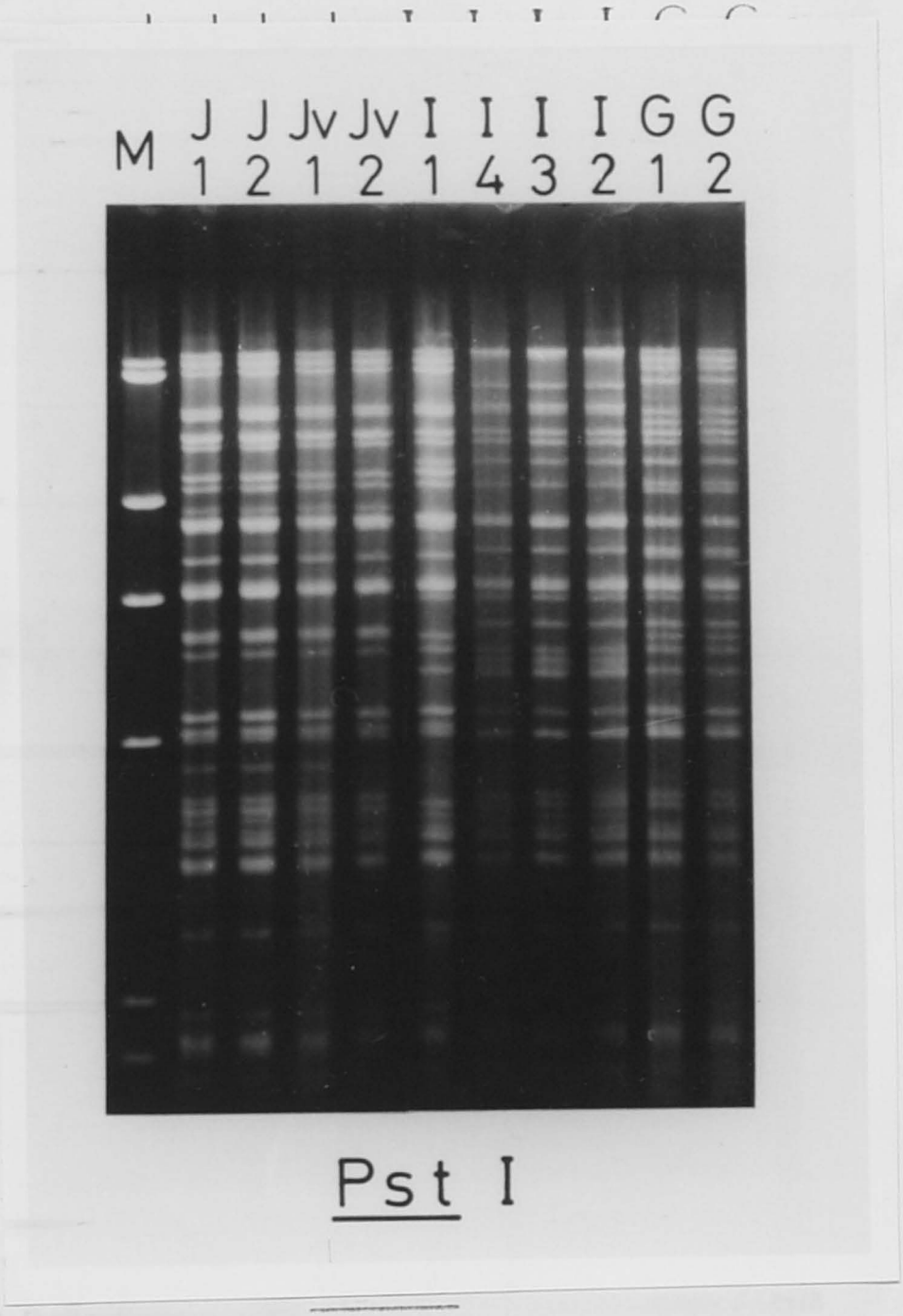


Fig. 19. PstI restriction fragment patterns of mtDNA from ten cultivars of Oryza. M: Lambda DNA digested with HindIII.

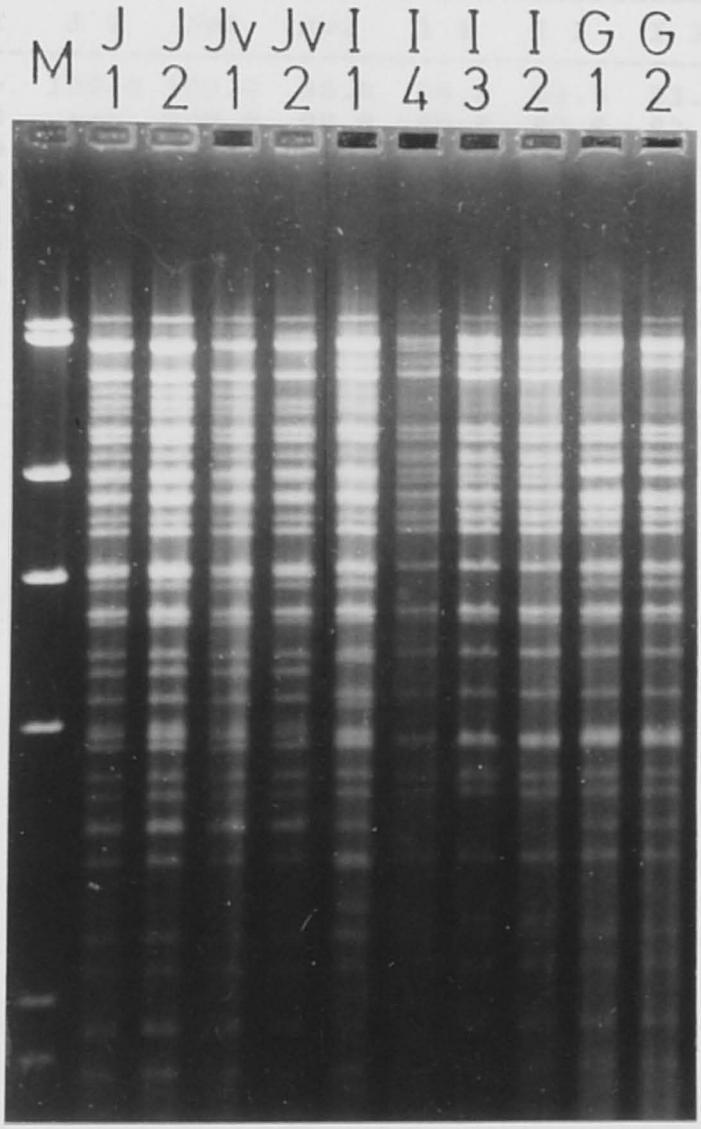


Fig. 20. PvuII restriction fragment patterns of mtDNA from ten cultivars of Oryza.

M: Lambda DNA digested with HindIII.

Table 18. Number of the total mtDNA fragments (below diagonal) and percentage of common mtDNA fragments (above diagonal) observed between ten cultivars of Oryza

Code	J 1	J 2	Jv1	Jv2	I 1	I 2	I 3	I 4	G 1	G 2
J 1.	---	100.0	100.0	98.8	89.8	83.5	83.5	83.5	83.9	83.9
J 2.	330	---	100.0	98.8	89.8	83.5	83.5	83.5	83.9	83.9
Jv1.	330	330	---	98.8	89.8	83.5	83.5	83.5	83.9	83.9
Jv2.	330	330	330	---	89.2	83.5	83.5	83.5	83.9	83.9
I 1.	334	334	334	334	---	86.1	86.1	86.1	86.4	86.4
I 2.	333	333	333	333	337	---	100.0	100.0	86.9	86.9
I 3.	333	333	333	333	337	336	---	100.0	86:9	86.9
I 4.	333	333	333	333	337	336	336	---	86.9	86.9
G 1.	341	341	341	341	345	344	344	344	---	100.0
G 2.	341	341	341	341	345	344	344	344	352	---

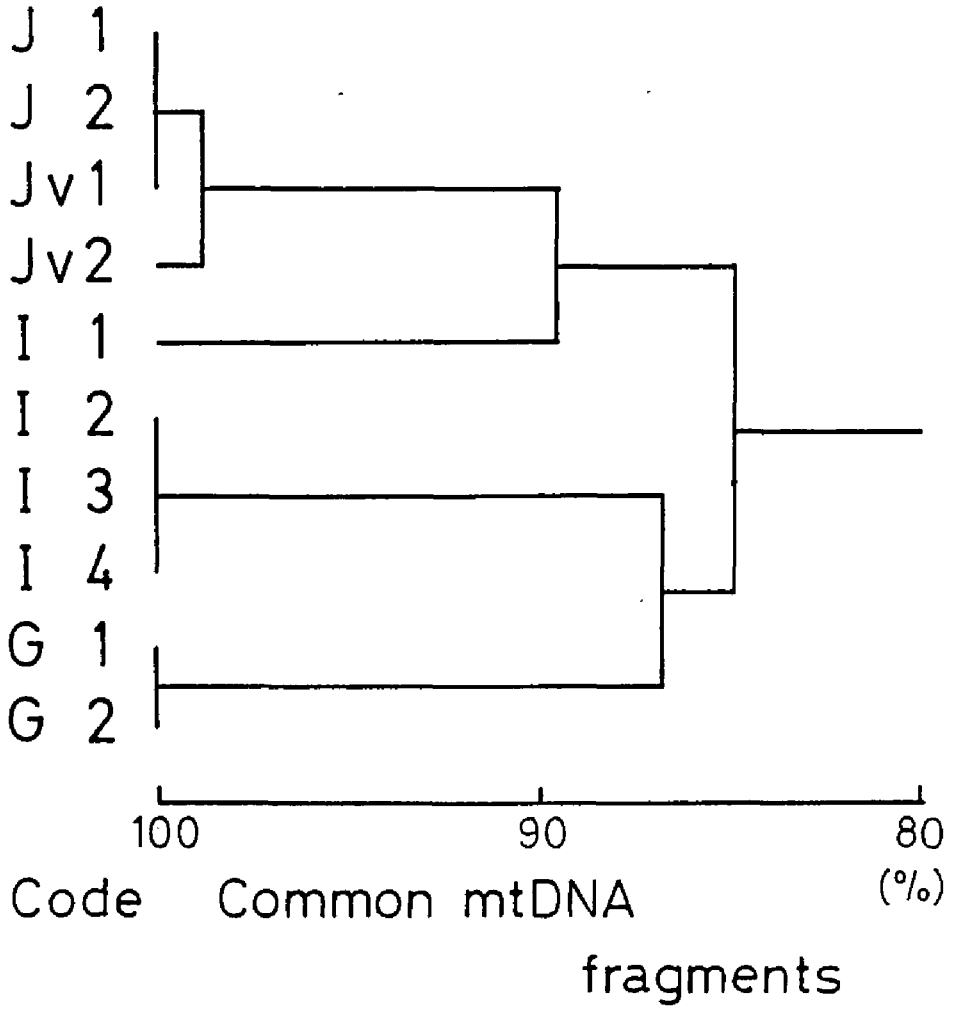


Fig. 21. Dendrogram showing genetic relationships between mitochondrial genomes of ten cultivars of Oryza based on the percentage of common mtDNA fragments observed in the restriction fragment patterns of their mtDNAs.

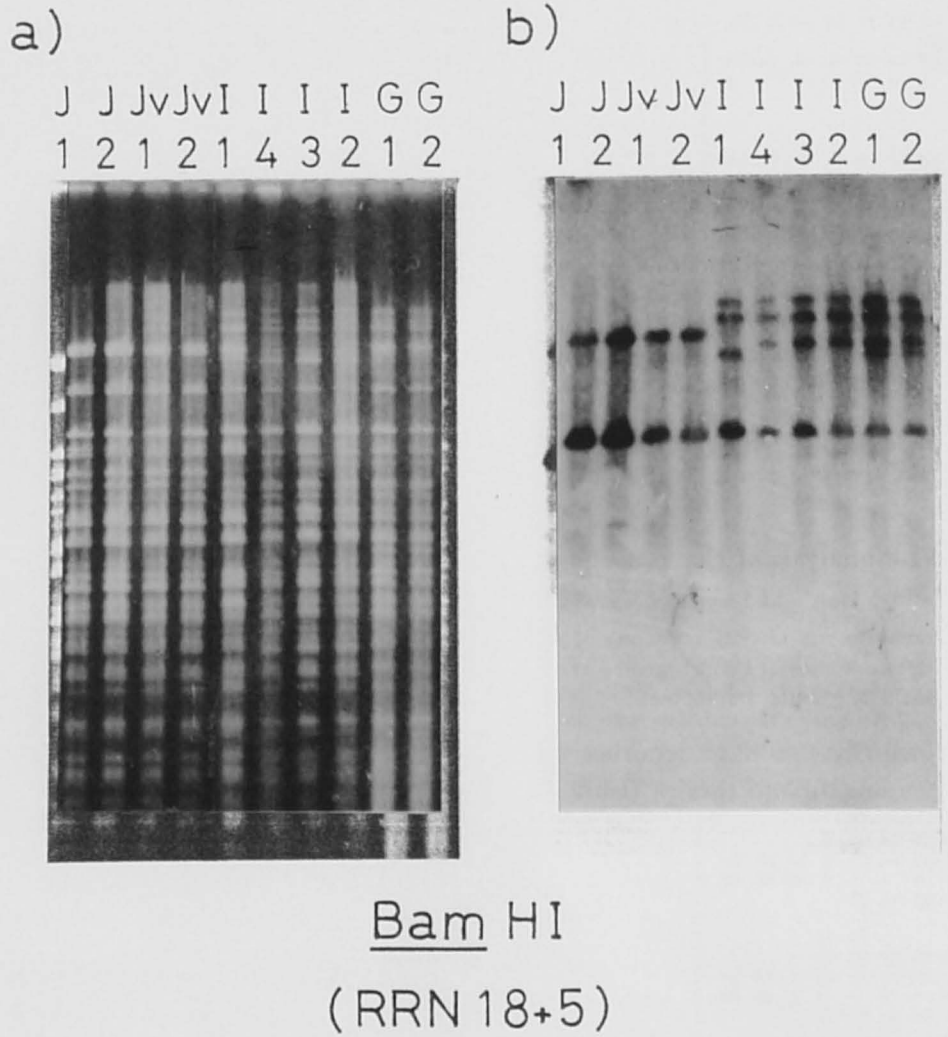


Fig. 22. a) BamHI restriction fragment patterns of mtDNA from ten cultivars of Oryza. b) Southern hybridization of the same patterns with wheat RRN18&5 probe.

Table 19 gives the number of the total fragments compared and the percentage of common fragments observed between ten cultivars. Their genetic relationships were shown by a dendrogram constructed by a UPGMA method applied for the percentage of common fragments (Fig. 23).

Table 19. Number of the total mtDNA fragments (below diagonal) and percentage of common mtDNA fragments (above diagonal) observed between ten cultivars of Oryza in Southern hybridization analysis

Code	J 1	J 2	Jv1	Jv2	I 1	I 2	I 3	I 4	G 1	G 2
J 1.	---	100.0	100.0	100.0	80.5	86.5	86.5	86.5	84.2	84.2
J 2.	72	---	100.0	100.0	80.5	86.5	86.5	86.5	84.2	84.2
Jv1.	72	72	---	100.0	80.5	86.5	86.5	86.5	84.2	84.2
Jv2.	72	72	72	---	80.5	86.5	86.5	86.5	84.2	84.2
I 1.	77	77	77	77	---	91.1	91.1	91.1	93.8	93.8
I 2.	74	74	74	74	79	---	100.0	100.0	97.4	97.4
I 3.	74	74	74	74	79	76	---	100.0	97.4	97.4
I 4.	74	74	74	74	79	76	76	---	97.4	97.4
G 1.	76	76	76	76	81	78	78	78	---	100.0
G 2.	76	76	76	76	81	78	78	78	80	---

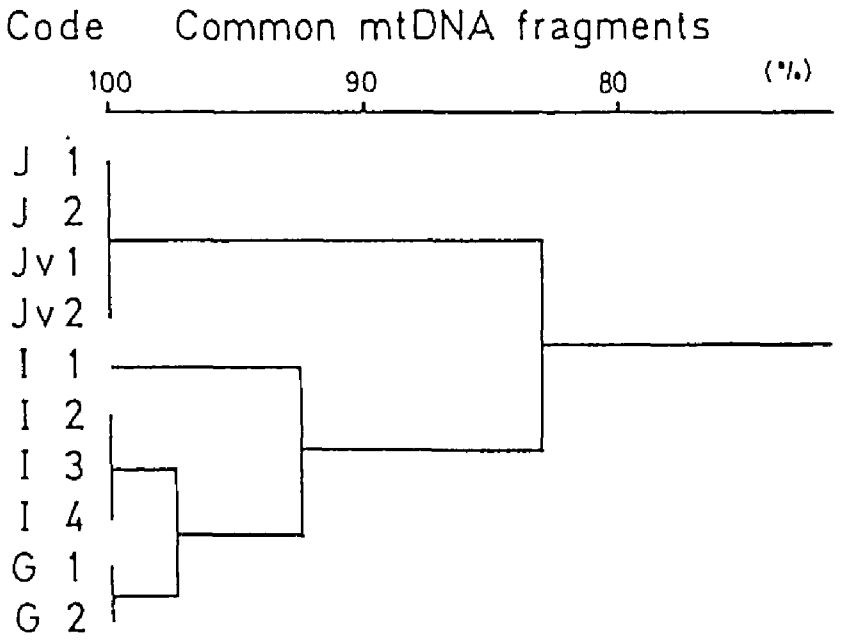


Fig. 23. Dendrogram showing genetic relationships between mitochondrial genomes of ten cultivars of Oryza based on the percentage of common fragments observed in their Southern hybridization patterns of their mtDNAs.

C. Nuclear genome differentiation in A-genome diploid species

Nuclear genome differentiation in A-genome diploid species

In order to reveal nuclear DNA variation in A-genome diploid species, Southern hybridization analysis on nuclear DNAs of 14 cultivars and 16 wild accessions (Table 4) were carried out. Their total DNAs were digested with EcoRI and HindIII. After electrophoresis, DNAs were transferred to Nylon membrane and hybridized with single-copy rice DNA probes. The probes were selected from 12 RFLP linkage groups, corresponding to different chromosomes (McCouch et al. 1988). Information on these probes is given in Table 20. In total, 24 combinations of hybridization patterns (2 enzymes X 12 probes) of 30 accessions were examined. Polymorphic fragment patterns were obtained in 19 out of 24 hybridization patterns. Fig. 24 is an example of the polymorphic fragment patterns.

All fragments were scored and genetic distances (Nei 1972) between all the accessions were calculated as follows:

$D = -\ln [J_{XY} / (J_X J_Y)^{1/2}]$,

where J_X, J_Y and J_{XY} are the arithmetic means, over all locl, of $\sum x_i^2$, $\sum y_i^2$ and $\sum x_i y_i$ (x_i and y_i are the frequencies of alleles of the ith locus in populations X and Y, respectively). In the present analysis, J_X and J_Y correspond to the numbers of total fragments in accessions X and Y, respectively, and J_{XY} is the number of common fragments observed between accessions X and Y.

The number of total fragments ranged from 22 to 31 among 30 accessions. Table 21 gives the genetic distances between 30 accessions, based on which a dendrogram was constructed using a complete linkage clustering method (Sneath and Sokal 1973) to show the differentiation of the nuclear genome among 30 accessions (Fig. 25).

Nuclear genome differentiation between O. sativa and O. glaberrima

Table 20. Probes used for Southern hybridization analysis of nuclear DNA

Probe	Size (kbp)	Vector	Cloning site	Chromosome no.[1]
RG236	1.4	pUC8	PstI	1 (1)
RG144	0.8	"	"	2 (2)
RG69	?	"	"	3 (4)
RG214	1.4	"	"	4 (12)
RG182	3.4	"	"	5 (5)
RG172	1.8	"	"	6 (3)
RG351	0.8	"	"	7 (7)
RG20	1.5	"	"	8 (8)
RG358	1.0	"	"	9 (9)
RG241	2.5	"	"	10 (10)
RG118	2.0	"	"	11 (11)
RG190	1.4	"	"	12 (6)

1) Previous chromosome numbers (McCouch et al. 1988) are given in parenthesis.

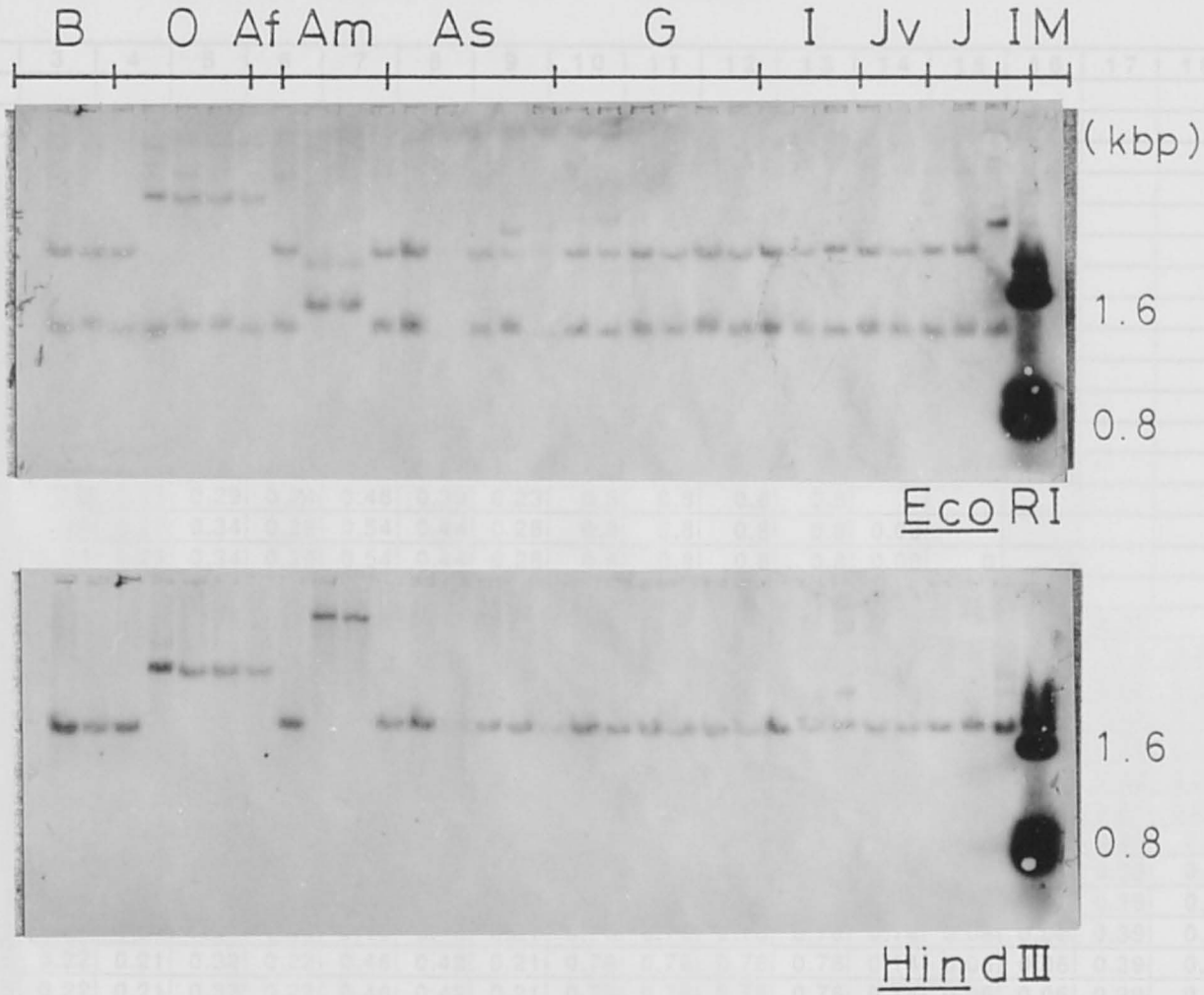


Fig. 24. Southern hybridization patterns of the EcoRI- and HindIII-digested total DNAs from 30 accessions of A-genome diploid species of Oryza, which were hybridized with a probe RG144. M: Molecular weight marker. As, Am, Af and O: O. perennis Asian, American, African and Oceanian form, respectively. B: O. breviligulata. J, Jv and I: O. sativa ecosp. Japonica, Javanica and Indica, respectively. G: O. glaberrima.

Table 21. Genetic distances (Nei 1972) between all pairs of 30 accessions of A-genome diploid species of Oryza

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	0.18																		
3	0.19	0.1																	
4	0.13	0.21	0.18																
5	0.11	0.27	0.19	0.09															
6	0.32	0.18	0.19	0.18	0.28														
7	0.58	0.55	0.52	0.52	0.52	0.52													
8	0.59	0.46	0.42	0.42	0.53	0.42	0.09												
9	0.32	0.17	0.18	0.17	0.27	0.13	0.52	0.42											
10	1.03	0.86	0.93	0.87	0.93	0.83	0.81	0.83	0.87										
11	1.03	0.86	0.93	0.87	0.93	0.83	0.81	0.83	0.87	0									
12	0.93	0.86	0.93	0.87	0.83	0.83	0.73	0.83	0.87	0.1	0.1								
13	0.93	0.86	0.93	0.87	0.83	0.83	0.73	0.83	0.87	0.05	0.05	0.05							
14	0.34	0.23	0.2	0.23	0.29	0.24	0.48	0.39	0.23	0.8	0.8	0.8	0.8						
15	0.39	0.27	0.24	0.28	0.34	0.29	0.54	0.44	0.28	0.8	0.8	0.8	0.8	0.08					
16	0.39	0.27	0.24	0.28	0.34	0.29	0.54	0.44	0.28	0.8	0.8	0.8	0.8	0.08	0				
17	0.21	0.28	0.25	0.19	0.17	0.39	0.66	0.61	0.39	0.94	0.94	0.94	0.94	0.35	0.4	0.4			
18	0.31	0.39	0.35	0.16	0.26	0.35	0.77	0.64	0.3	1.01	1.01	1.01	1.01	0.42	0.48	0.48	0.32		
19	0.24	0.32	0.29	0.1	0.2	0.29	0.67	0.56	0.23	1	1	1	1	0.35	0.41	0.41	0.3	0.06	
20	0.48	0.35	0.32	0.37	0.48	0.37	0.66	0.6	0.31	0.87	0.87	0.87	0.87	0.33	0.33	0.33	0.56	0.52	0.44
21	0.3	0.38	0.35	0.19	0.3	0.4	0.79	0.65	0.35	1.08	1.08	1.08	1.08	0.48	0.55	0.55	0.42	0.18	0.21
22	0.16	0.24	0.2	0.1	0.2	0.3	0.71	0.59	0.29	1.08	1.08	1.08	1.08	0.37	0.42	0.42	0.22	0.23	0.17
23	0.13	0.12	0.09	0.2	0.17	0.21	0.44	0.46	0.2	0.91	0.91	0.81	0.81	0.22	0.27	0.27	0.32	0.39	0.32
24	0.15	0.23	0.15	0.1	0.11	0.29	0.67	0.56	0.28	1.11	1.11	1.11	1.11	0.3	0.35	0.35	0.21	0.22	0.16
25	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33
26	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33
27	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33
28	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33
29	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33
30	0.37	0.25	0.22	0.21	0.32	0.22	0.46	0.42	0.21	0.78	0.78	0.78	0.78	0.14	0.06	0.06	0.39	0.4	0.33

Note) Accession numbers (see Table 4) are indicated in the top row and the left end column

Table 21. (continued)

	20	21	22	23	24	25	26	27	28	29	30
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											
21	0.35										
22	0.47	0.22									
23	0.35	0.39	0.23								
24	0.44	0.21	0.12	0.22							
25	0.26	0.47	0.35	0.25	0.33						
26	0.26	0.47	0.35	0.25	0.33	0					
27	0.26	0.47	0.35	0.25	0.33	0	0				
28	0.26	0.47	0.35	0.25	0.33	0	0	0			
29	0.26	0.47	0.35	0.25	0.33	0	0	0	0		
30	0.26	0.47	0.35	0.25	0.33	0	0	0	0	0	

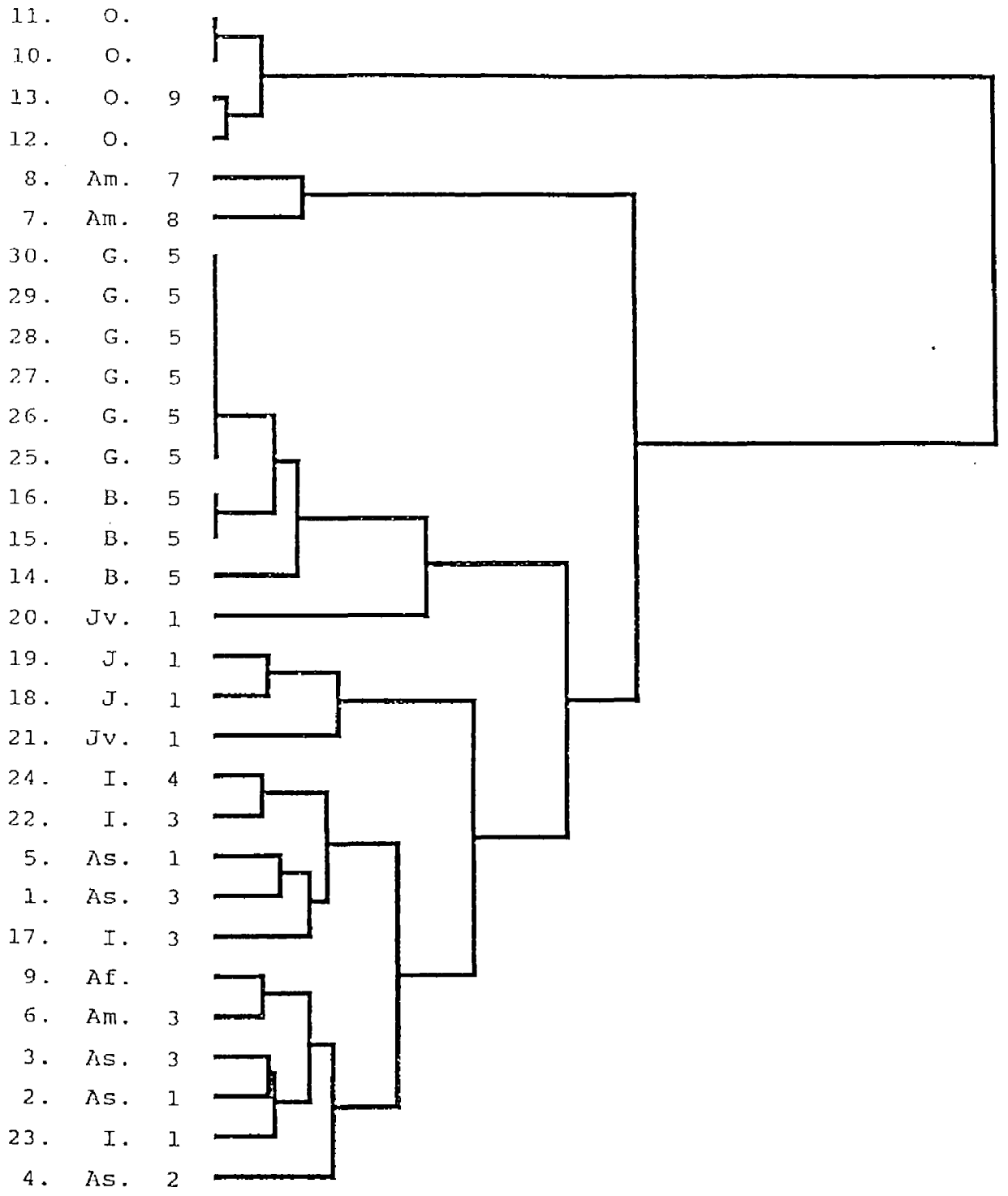


Fig. 25. Dendrogram showing genetic relationships of nuclear genome between 30 accessions of A-genome diploid species of Oryza. The first column on the left gives accession number (see Table 4), the second column abbreviated taxon (see Fig. 24) and the third column chloroplast genome type of these accessions.

Eight cultivars of O. sativa (J1, J2, Jv1, Jv2, I1, I2, I3 and I4) and two cultivars of O. glaberrima (G1 and G2) were selected from the materials given in Table 2 for Southern hybridization analysis of nuclear DNA. Their total DNAs were digested with HindIII and PstI. After electrophoresis, DNAs were transferred to Nylon membrane and hybridized with 18 nuclear DNA probes prepared from O. sativa cv. Nipponbare and IR36 (Table 22). Polymorphic fragment patterns were obtained in 12 out of 31 combinations of probe-endonucleases (Table 22). Fig. 26 shows an example of the polymorphic fragment patterns. All fragments were scored and the percentage of common fragments between different accessions was used as an index of the similarity in their nuclear DNAs. The total number of fragments and the percentage of common fragments observed between ten cultivars are given in Table 23, based on which a dendrogram is drawn by a UPGMA method that indicates genetic relationships between nuclear genomes of the cultivars (Fig. 27).

Nuclear genome differentiation in O. sativa

Table 3 gives 115 cultivars of O. sativa from 17 Asian countries, of which isozyme constitutions were already analyzed by Glaszmann (1985). In order to confirm whether the materials are the same as those used by Glaszmann, their isozyme constitutions as to five loci, i.e., Pgi-1, Pgi-2, Amp-1, Amp-2 and Amp-3, were examined. In most cultivars except ten, the identical isozyme constitutions were confirmed. As for the ten exceptional cultivars, a plant which showed most similar constitution to those described by Glaszmann was selected among several plants as a representative of the cultivar. Table 24 shows the comparison of the isozyme constitutions of ten exceptional cultivars between Glaszmann's data and the present results.

Of 115 cultivars listed in Table 3, 112 cultivars (No. 1-112) were used for Southern hybridization analysis of nuclear DNA. Their total DNAs were digested with EcoRI and HindIII. After electrophoresis, DNAs were transferred to Nylon membrane and hybridized with 12 single-copy rice DNA probes, which were the same as used in a previous study (Table 20). In total, 24 combinations of hybridization (2 endonucleases X 12 probes) were analyzed. Polymorphism was

Table 22. Polymorphism of Southern hybridization patterns of total DNAs of ten cultivars probed with 18 nuclear DNA clones

Clone no.	Origin[1]	Vector	Cloning site[2]	Size (kbp)	Hybrid. pattern[3] HindIII	Hybrid. pattern[3] PstI
1.	N	pUC119	P	1.0	P	M
2.	"	"	"	1.1	P	M
3.	"	"	"	1.4	P	M
4.	"	"	"	1.6	P	M
5.	"	"	"	2.5	M	M
6.	"	"	"	2.8	--	M
7.	"	"	"	3.7	P	P
8.	"	"	"	3.9	M	M
9.	"	"	"	4.8	M	M
10.	"	"	"	10.5	P	M
11.	"	"	"	12.6	M	M
12.	I	"	H	1.4	--	M
13.	"	"	"	3.1	--	M
14.	"	"	"	5.0	P	P
15.	"	"	"	5.2	P	P
16.	"	"	P	2.1	--	M
17.	"	"	"	2.7	P	M
18.	"	"	"	4.7	--	M

1) N: Nipponbare, I: IR36.
2) P: PstI site, H: HindIII site.
3) M: Monomorphic, P: Polymorphic.
--: Not tested.

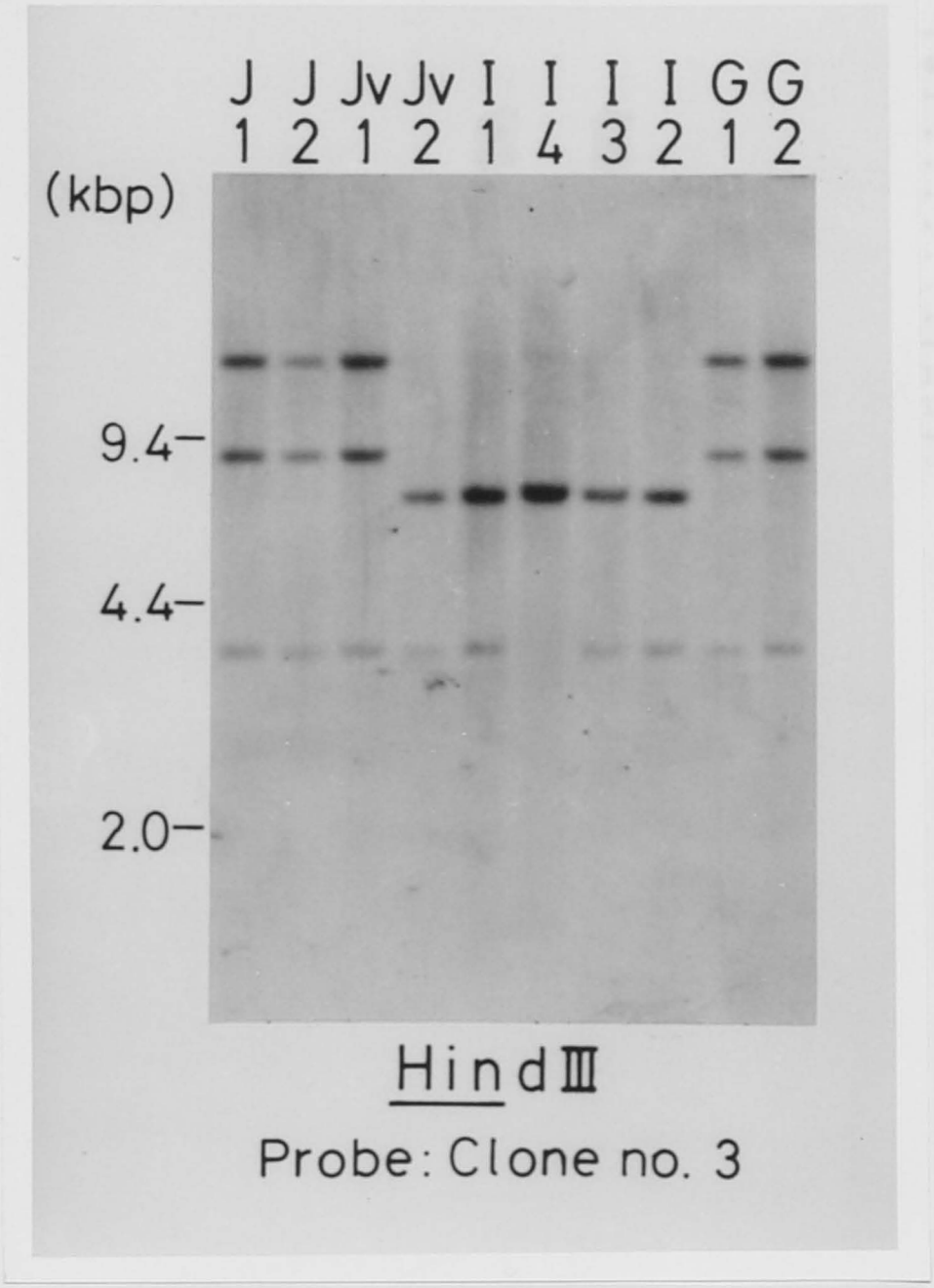


Fig. 26. Southern hybridization patterns of the HindIII-digested total DNA from ten cultivars of Oryza probed with no. 3 nuclear DNA clone.

Table 23. Number of the total fragments (below diagonal) and percentage of common fragments (above diagonal) observed between nuclear DNAs of ten cultivars of Oryza

Code	J 1	J 2	Jv1	Jv2	I 1	I 2	I 3	I 4	G 1	G 2
J 1.	---	100.0	100.0	95.9	94.2	90.9	90.8	81.4	88.3	86.7
J 2.	122	---	100.0	95.9	94.2	90.9	90.8	81.4	88.3	86.7
Jv1.	122	122	---	95.9	94.2	90.9	90.8	81.4	88.3	86.7
Jv2.	121	121	121	---	98.3	95.0	91.5	85.7	84.0	84.0
I 1.	121	121	121	120	---	95.0	93.2	85.7	82.4	82.4
I 2.	121	121	121	120	120	---	88.1	87.5	79.0	79.0
I 3.	119	119	119	118	118	118	---	89.1	88.9	87.2
I 4.	113	113	113	112	112	112	110	---	79.3	79.3
G 1.	120	120	120	119	119	119	117	111	---	98.3
G 2.	120	120	120	119	119	119	117	111	118	---

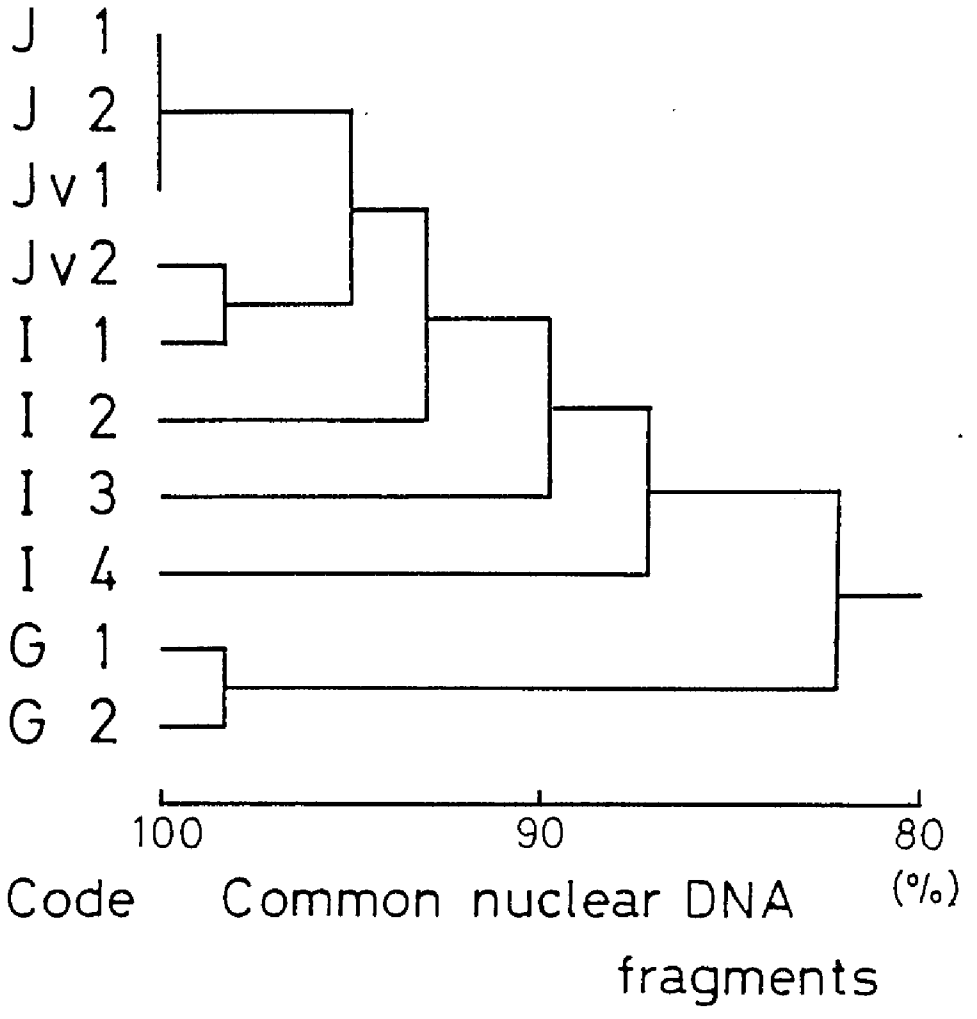


Fig. 27. Dendrogram showing genetic relationships between nuclear genomes of ten cultivars of Oryza based on the percentage of common nuclear DNA fragments.

Table 24. Comparison of the isozyme constitutions between Glaszmann's data and the present results of ten cultivars of O. sativa

No.	Acc.no.	Glaszmann's data[1]					Present results				
		A	B	C	D	E	A	B	C	D	E
24	7722	1	1	2	2	2	1	2	2	2	2
30	12881	2	4	0	1	2	2	4	0	1	1
39	27590	2	1	6	1	2	2	1	6	1	2
40	27762	1	1	5	2	1	2	2	5	2	1
41	27798	2	1	0	1	1	2	1	0	1	2
56	43394	2	1	1	1	1	2	1	1	0	1
73	58930	2	4	4	1	2	1	1	1	2	1
97	26872	2	1	1	1	1	2	3	2	2	1
106	32368	2	4	0	1	1	2	4	0	1	2
111	46768	2	1	0	1	1	1	1	0	1	1

1) A, B, C, D and E indicate the loci of Pgi-1, Pgi-2, Amp-3, Amp-2 and Amp-1, respectively. Allele nomenclature follows to Glaszmann et al. (1988).

revealed in 17 out of 24 hybridization patterns. Fig. 28 shows an example of the polymorphic fragment patterns.

All fragments were scored and genetic distances (Nei 1972) between all the cultivars were calculated. The number of total fragments ranged from 25 to 31 among 112 cultivars. Table 25 shows the genetic distances between 112 cultivars. Based on Table 25, a dendrogram is constructed by a complete linkage clustering method to show the differentiation of the nuclear genome among 112 cultivars (Fig. 29).

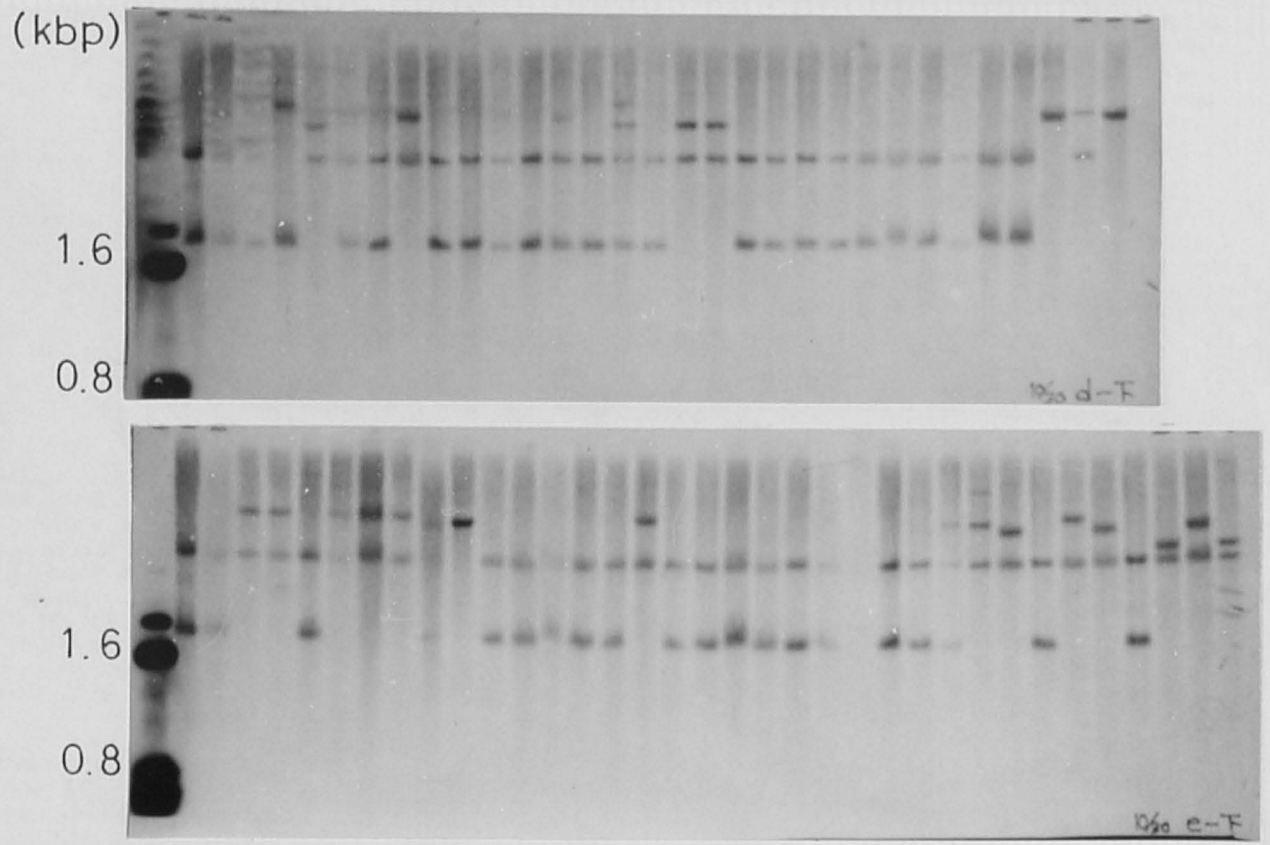
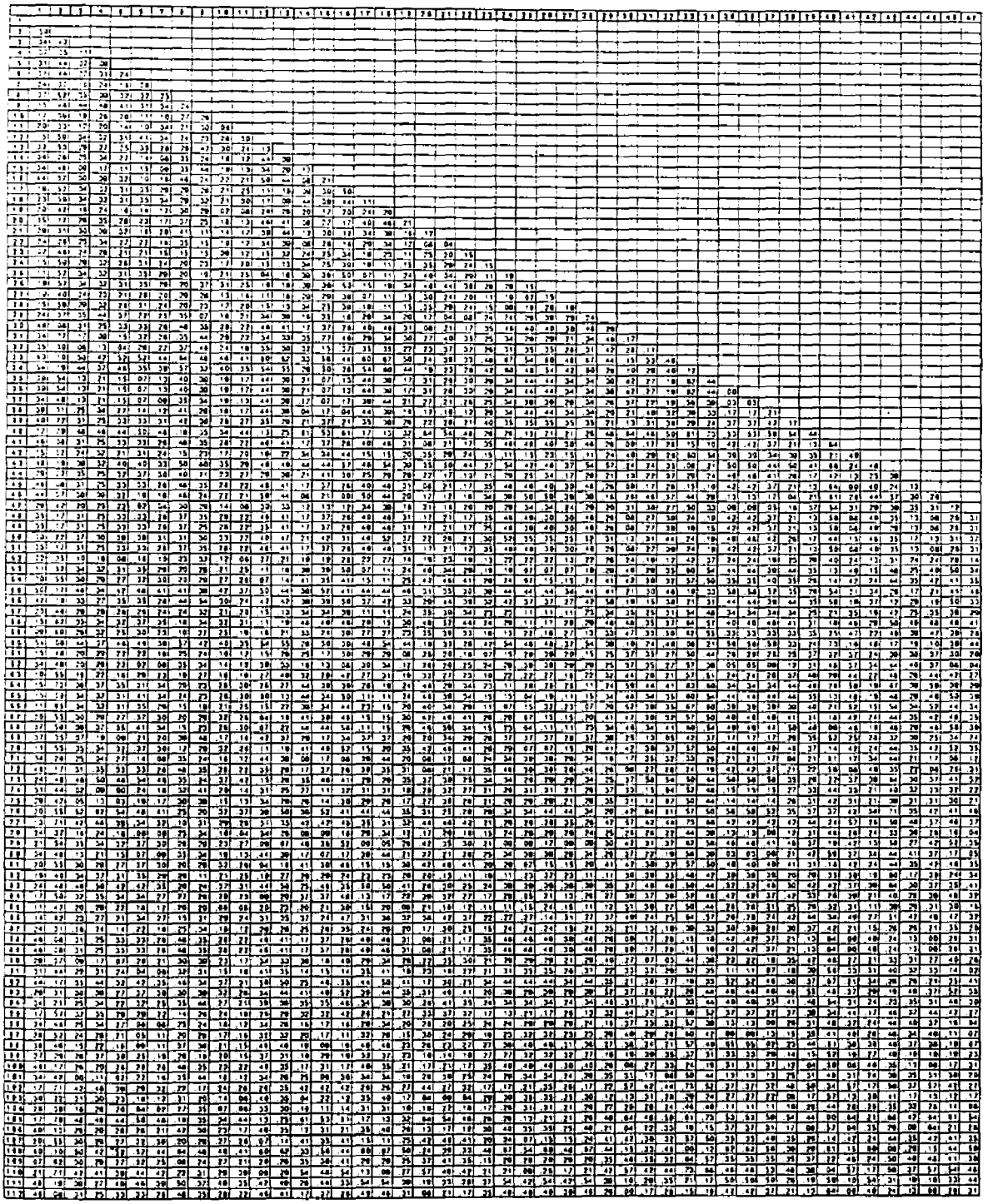


Fig. 28. Southern hybridization patterns of the EcoRI-digested total DNA of O. sativa, that was hybridized with a probe RG214.

Table 25. Genetic distances (Nei 1972) between the nuclear genomes of all pairs of 112 cultivars in O. sativa



Note) Accession numbers (see Table 3) are indicated in the top row and the left column

Table 25. (continued)

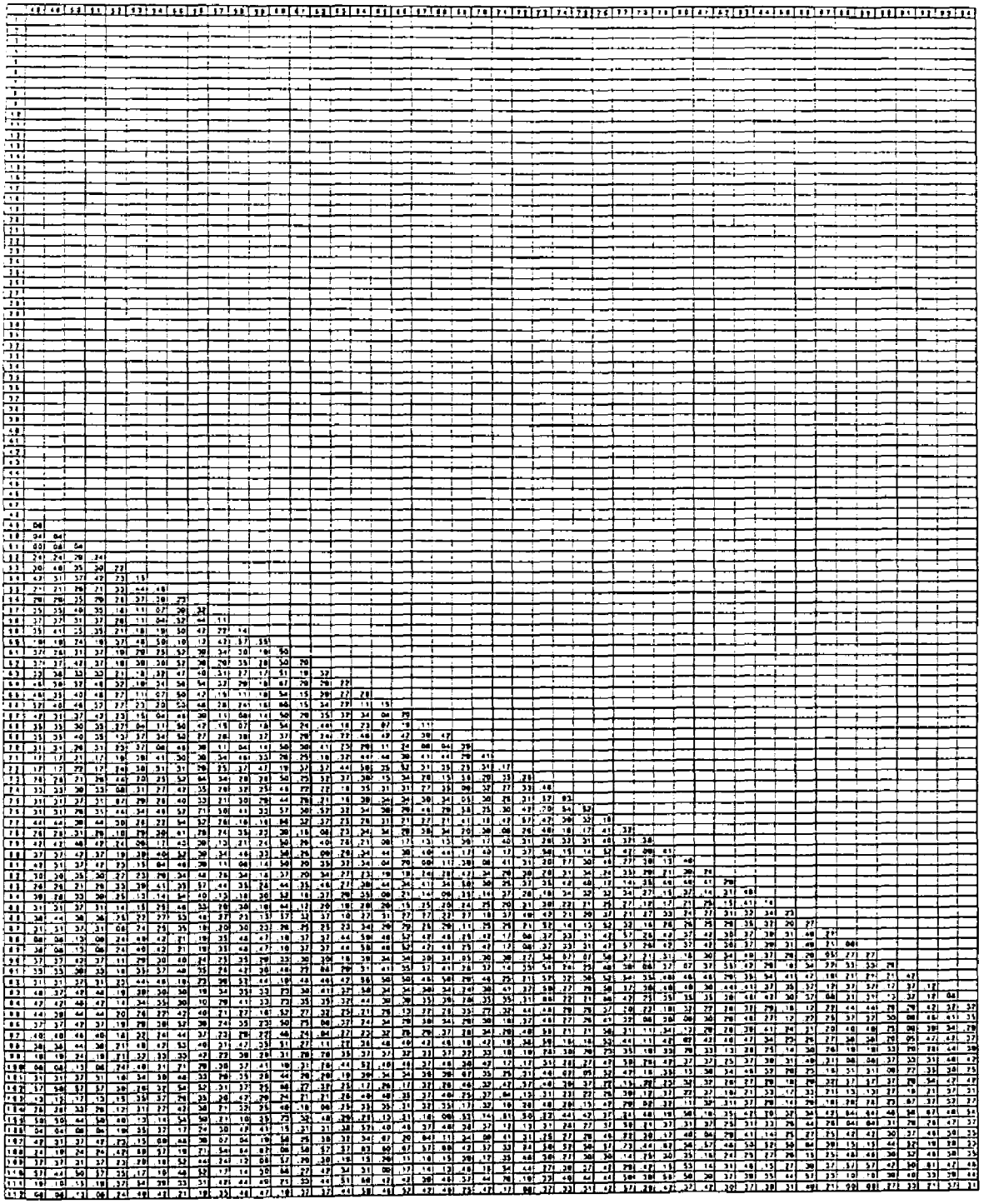
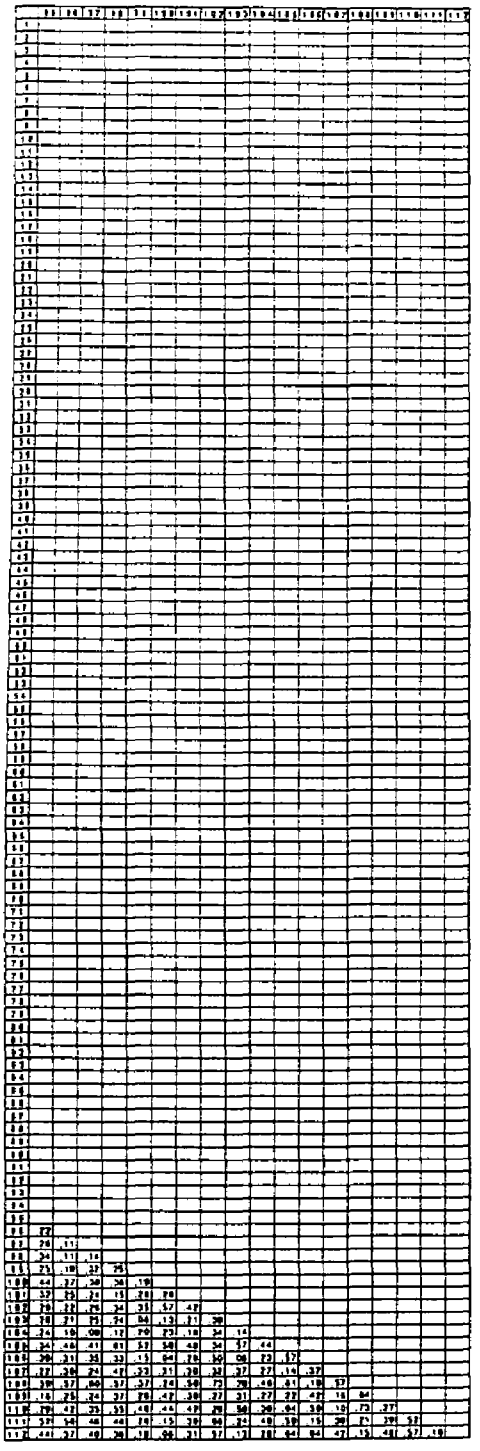


Table 25. (continued)



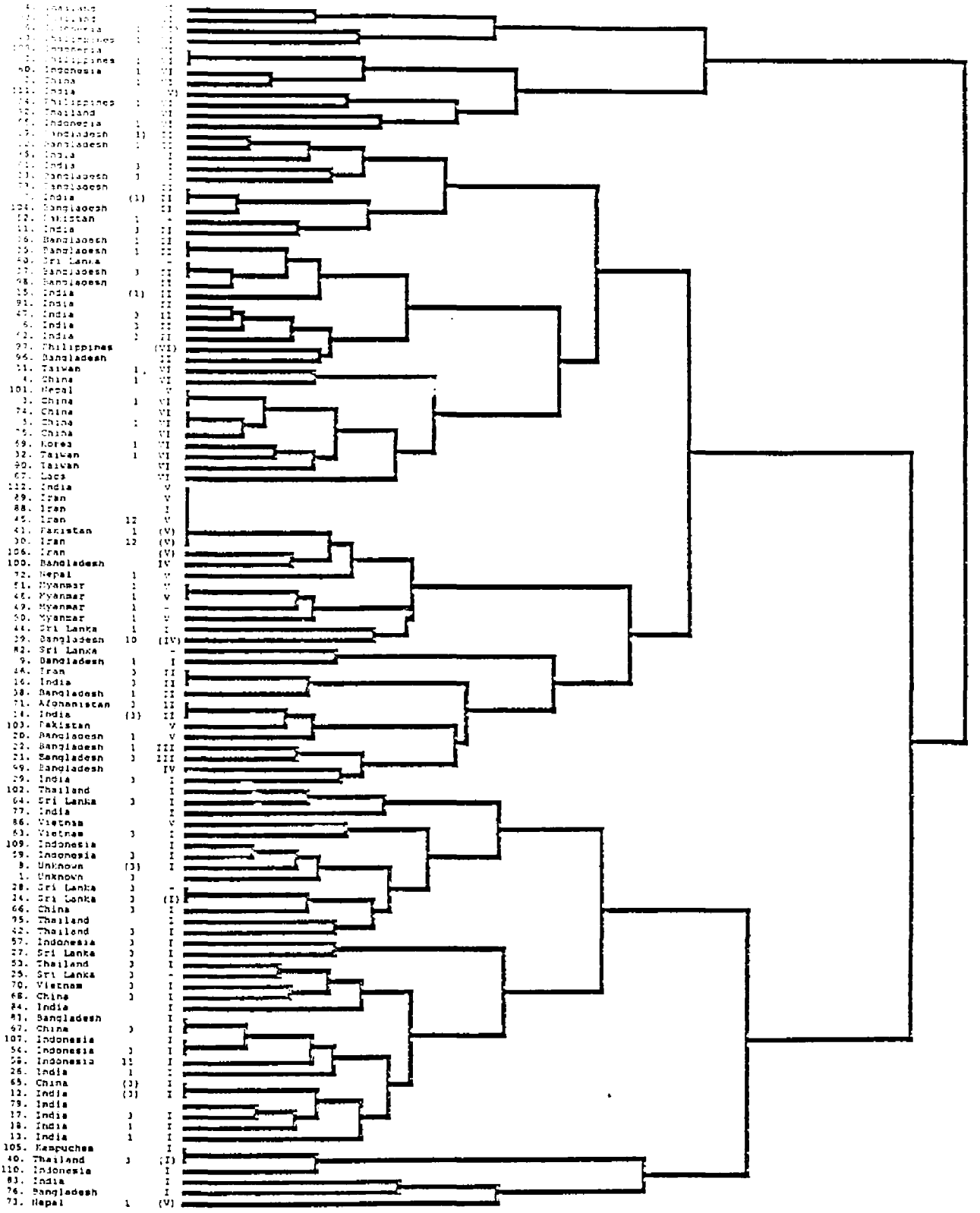


Fig. 29. Dendrogram showing genetic relationships of nuclear genome between 112 cultivars O. sativa as revealed by RFLP analysis of their nuclear DNAs. The first column gives their accession number (see Table 3), the second column origin, the third column chloroplast genome type and the forth column enzymatic group (Glaszmann 1985). Parentheses in the third and forth columns indicate putative types and groups with incomplete data respectively.

IV. DISCUSSION

Chloroplast genome differentiation in wild A-genome species

Restriction endonuclease analysis of ctDNA with EcoRI, HindIII and PstI revealed that O. perennis contained seven chloroplast genome types, whereas O. breviligulata had only one. Ichikawa et al. (1986) studied BamHI restriction fragment patterns of ctDNAs isolated from various wild Oryza species having different nuclear genome constitutions, such as AA, BB, BBCC, CC, CCDD and EE, and found many differences between them. These differences were larger than those found above. Yet, chloroplast genome differentiation in O. perennis appears to be extensive, and seems to be related to its wide geographical distribution.

The African and Oceanian forms of O. perennis had one chloroplast genome type each (type 6 and 9, respectively). In contrast, the Asian and American forms had three (type 1, 2 and 3) and four types (type 3, 6, 7 and 8), respectively. Three types of the Asian form were similar in their restriction fragment patterns of ctDNA to each other, whereas the four types of American form were rather different one another. According to Barnes and Pental (1985), repeated DNA sequences and ribulose 1,5-bisphosphate carboxylase/oxygenase in some accessions of the American form of O. perennis, are quite different from those of O. sativa, O. glaberrima and other accessions of O. perennis. Their and the present results indicate that the American form of O. perennis has differentiated from other forms to great extent. A single accession of the American form had type 3 chloroplast genome, which is the major chloroplast genome type of the Asian form, and is very different from the chloroplast genomes of other forms. It is difficult to understand how a single accession of the American form that evolved independently from the Asian form has the same type 3 chloroplast genome as the most accessions of the latter. Second (1985) proposed a hypothesis, based on the results of isozyme analysis, that some accessions of the American form were recently introduced and colonized in the present habitat. The present results support his hypothesis.

Chloroplast genome relationships between wild and cultivated species

It is a well-known fact that the chloroplast genome is evolutionarily very conservative at the species level (Ogihara and Tsunewaki 1982; Doebley et al. 1987). Therefore, if wild and cultivated species share the same chloroplast genome type exclusively, the latter can be assumed to have originated from the former.

Using three endonucleases, i.e., EcoRI, HindIII and PstI, the chloroplast genome of cultivated rice species could be classified into four types, 1, 3, 4 and 5. O. sativa contains three chloroplast genome types, 1, 3 and 4, whereas O. glaberrima has only one (type 5). This suggests that the chloroplast genome has already differentiated between O. sativa and O. glaberrima. In O. sativa, the chloroplast genome has also differentiated. O. sativa ecosp. Japonica and Javanica contain the same chloroplast genome (type 1), and ecosp. Indica has three genome types (type 1, 3 and 4). The chloroplast genome differentiation within ecosp. Indica is greater than that between ecosp. Japonica and Javanica. As compared with O. perennis, the chloroplast genome differentiation in O. sativa is small. This suggests that O. sativa has originated from local populations of O. perennis.

As stated above, O. sativa contains three chloroplast genomes (type 1, 3 and 4), and O. glaberrima only one (type 5). The type 5 chloroplast genome was found in all accessions of O. breviligulata, but not in other species. This fact strongly suggests that O. glaberrima originated from O. breviligulata, supporting the conclusions of Morishima et al. (1963) and Second (1982). Two chloroplast genome types, 1 and 3, the most common in O. sativa, were found in the Asian form of O. perennis. A single accession of the American form also had the type 3 chloroplast genome. As mentioned earlier, this type is exceptional among the accessions of American form. These results suggest that O. sativa originated from the Asian form of O. perennis. In addition, the type 4 chloroplast geneme found in a single accession of O. sativa seems to have derived secondarily from the type 3 chloroplast genome (Fig. 30).

The domestication of O. sativa has been proposed to be either monophyletic (Oka and

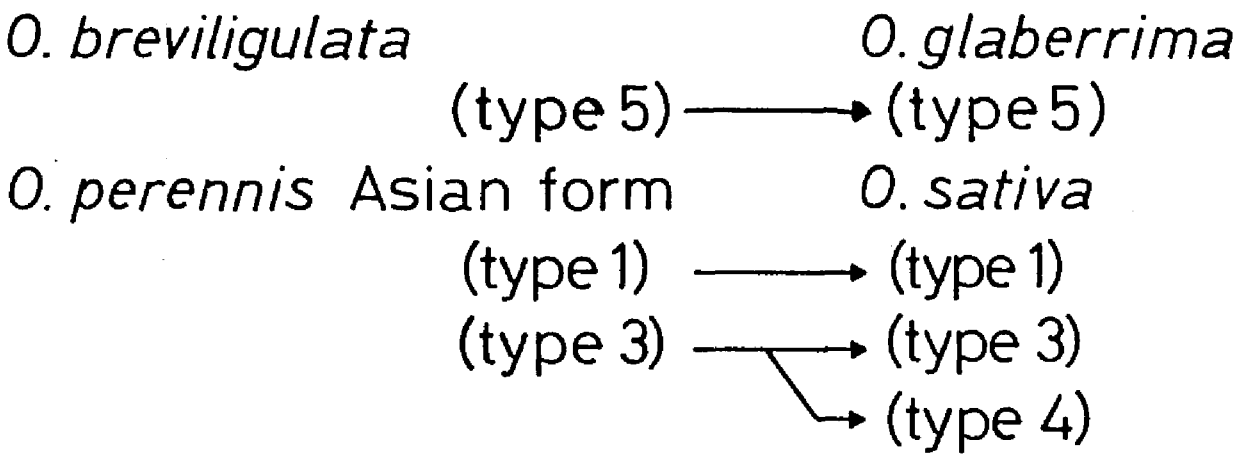


Fig. 30. Possible origins of two cultivated species.

Chang 1962) or diphyletic (Second 1982). The above results support the latter hypothesis, since the type 1 and 3 chloroplast genomes found in O. sativa are likely to have originated independently from the corresponding types in the Asian form of O. perennis.

Molecular differences between three major chloroplast genomes found in cultivated species

Nineteen accessions (Table 2) selecteed from the cultivated A-genome species, i.e., O. sativa ecosp. Japonica, Javanica and Indica, and O. glaberrima, were classified into three chloroplast genome types, type 1, 3 and 5 from the restriction fragment patterns of their ctDNAs treated with 11 restriction endonucleases. All cultivars of O. sativa ecosp. Japonica and Javanica had the type 1 genome, and no variation was observed among them. Most of O. sativa ecosp. Indica possessed the type 3 chloroplast genome. All cultivars of O. glaberrima had the type 5 chloroplast genome.

As all the restriction endonucleases employed in the present investigation recognize specific six-base-pair sequence, the total number of the fragments observed in each digestion corresponds to the total number of the six-base sequences cleaved with this endonuclease. Table 26 shows the total number of the restriction sites recognized, and the number of the mutational events assumed between the three chloroplast genome types. In total, 1194 bp were examined and three, four and seven mutational events were found between the chloroplast genome type 1 and 3, 1 and 5, and 3 and 5, respectively. If the type 1 genome is assumed to be the root, the type 3 and 5 genomes have differentiated to the opposite directions from each other. Probably the differentiation had taken place in wild A-genome species, and the resulted chloroplast genomes were transferred to cultivated species.

Three cultivars were selected as the representative of each genome, and the P11, P13 and P14 fragments of their ctDNAs were partially sequenced (Fig. 8). The nucleotide sequences of 1738 bp, in total, were compared between them. However, no difference was detected. Sequence homology between rice and other plants, i.e., maize (McIntosh et al. 1980; Krebbers et al. 1982; Rodermel and Bogorad 1987), wheat (Howe et al. 1982, 1985; Terach et al. 1987) and tobacco

Table 26. The total number of the sequences recognized with 11 endonucleases and the number of the mutational events between three chloroplast genomes, type 1, 3 and 5

Endonuclease	Bam HI	Bql II	Eco RI	Eco RV	Pvu II	Sal I	Sma I	Xho I	Hin dIII	Pst I	Xba I	Total
No. fragments	21	23	22	24	11	10	14	19	23	15	17	199
No. bases	132	138	132	144	66	60	84	114	138	90	102	1194
No. mutational events between												
Type 1 and 3	0	1	1	0	0	0	0	0		1[1)]		3
Type 1 and 5	0	1	3	0	0	0	0	0		0		4
Type 3 and 5	0	2	4	0	0	0	0	0		1[1)]		7

1) A 0.1 kbp deletion is common in HindIII, PstI and XbaI digests.

(Shinozaki and Sugiura 1982; Shinozaki et al. 1983; Deno et al. 1984) was examined. The coding regions revealed 97, 96 and 89 % homology between rice and three other plants, maize, wheat and tobacco, respectively, whereas sequence homology in flanking non-coding regions were low.

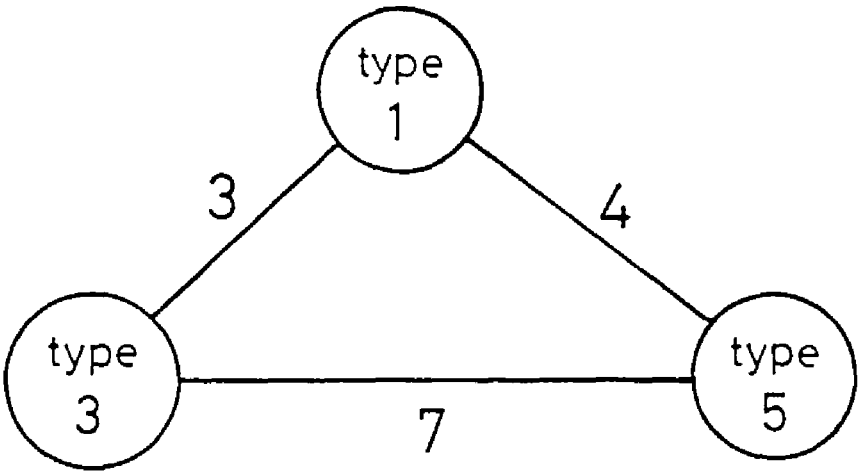
So far as the present results are concerned, restriction endonuclease analysis proved to be more effective to detect ctDNA variation than nucleotide sequence analysis, because seven mutational events were discovered in 1194 bp by the former method, whereas no mutation was found in 1738 bp which were sequenced (Fig. 31). Their difference in detecting ctDNA variation can be explained by assuming that restriction endonuclease analysis picked up variations in different regions of ctDNA molecule, whereas sequence analysis was applied to a specific region of relatively high conservatism.

Chloroplast genome differentiation in O. sativa

Among 68 cultivars of O. sativa examined, five chloroplast genome types (types 1, 3, 10, 11 and 12) were recognized. Their differences were ascribed to two base substitutions and four length mutations. SmaI and HindIII site changes were found in the P5 fragment in the small single copy region and in the P1 fragment in the large single copy region, respectively. As for length mutations, three 0.1 kbp and a 0.3 kbp change were detected in the P1, P2 and P12 fragments in the large single copy region flanking an inverted repeat sequence (Fig. 17). Similar cases were reported in related genera, such as Zea (Doebley et al. 1987), Triticum and Aegilops (Ogihara and Tsunewaki 1988) and Avena (Murai and Tsunewaki 1987). In Triticum and Aegilops, Ogihara et al. (1988) determined the nucleotide sequences of the region involved in two size changes and found that illegitimate recombinations between short direct repeats caused these changes. In Oryza, Moon et al. (1987, 1988) reported that a similar event occurred near the region of the rbcL gene. Therefore, such a mechanism might have been involved in the fragment length mutations detected in the present investigation on chloroplast genome differentiation in O. sativa.

In the previous work, the type 1 and 3 chloroplast genomes were also found among wild A-genome species. This suggests differentiation between the type 1 and 3 chloroplast genomes

a) Restriction endonuclease analysis (1194 bp)



b) Sequence analysis (1738 bp)

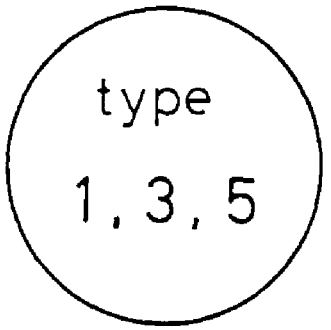


Fig. 31. Comparison of the results between restriction endonuclease analysis and sequence analysis with type 1, 3 and 5 chloroplast genomes.

had already occurred before rice cultivation. The type 10, 11 and 12 chloroplast genomes were found in only four out of 68 cultivars of O. sativa. The differences between these genomes and the type 1 or 3 were ascribed to four mutations; two mutations from the type 3 to the type 10, and two from the type 1 to the type 12, including one from the type 1 to the type 11. These mutations are assumed to have occurred since rice was cultivated. On the other hand, most cultivars have either type 1 or 3 chloroplast genome. These facts indicate strong evolutionary conservatism of the chloroplast genome in O. sativa.

Geographical distribution of different chloroplast genomes in O. sativa

As stated above, five chloroplast genome types, i.e., two major (type 1 and 3) and three minor types (type 10, 11 and 12), were found among 68 cultivars in O. sativa. The type 11 and 12 chloroplast genomes were apparently derived from the type 1, whereas the type 10 originated from the type 3 genome (Fig. 17). The type 1 and 3 genomes are the representative of Japonica and Indica, respectively (Ishii et al. 1988). Their differences can be explained by two length mutations, namely, one insertion and one deletion, both of which are irreversible. Based on these facts, 68 cultivars can be divided into Japonica (type 1, 11 and 12) and Indica (type 3 and 10) groups based on their chloroplast genomes. Cultivars from east Asia, such as Philippines, Taiwan and Korea, as well as Japan (Ishii et al. 1988), have only the Japonica chloroplast genomes. In other areas of Asia, chloroplast genomes of both Japonica and Indica groups are frequently found, where no clear geographical differentiation of chloroplast genome in rice cultivars was noticed.

Mitochondrial genome differentiation in cultivated species, and comparison between mitochondrial and chloroplast genome differentiation

Based on the results of restriction endonuclease and Southern hybridization analyses with ten cultivars, two dendrograms showing genetic relationships between O. sativa and O. glaberrima were constructed (Figs. 21 and 23). In both dendrograms ten cultivars were divided into four groups, one consisting of Japonica and Javanica cultivars, two of Indica cultivars, and one of O.

glaberrima cultivars. However, relationships between four groups were somewhat different between the dendrograms. Especially, the percentage of similarity between the Indica (I2, I3 and I4) and O. glaberrima group (G1 and G2) was 87 % from the results of restriction endonuclease analysis, whereas it was 97 % from Southern hybridizaion analysis. A possible reason for this discrepancy is a small number of probes used in Southern hybridization analysis. The number of the fragments compared in Southern hybridization analysis was much smaller than that in restriction endonuclease analysis. Here, the results of restriction endonuclease analysis will be taken to represent mitochondrial genome differentiation.

From the restriction endonuclease analysis of mtDNA, five mitochondrial gemone types were observed, which were grouped into three main clusters (Fig. 21). These clusters well correspond to those of chloroplast genome (Fig. 7). The percentages of the common mtDNA fragments between these clusters range from 85 % to 87 %. However, no differences were observed among two Japonica and one Javanica cultivar (J1, J2 and Jv1), among three Indica cultivars (I2, I3 and I4), and between two O. glaberrima cultivars (G1 and G2). This fact suggests that mtDNA was also conservative during speciation in cultivated rice.

As to the relationship between chloroplast and mitochondrial genome differentiation, most cultivars shared the same chloroplast and mitochondrial genome types. This coincidence seems to reflect their coevolution through maternal inheritance. But two cultivars (Jv2 and I1) had different mtDNA from that of three other cultivars (J1, J2 and Jv1), all of which have identical chloroplast genome. On the other hand, three Indica cultivars (I2, I3 and I4) showed no difference in their mtDNA but differed in their ctDNA. These results confirm that chloroplast and mirochondrial genome differentiation are basically independent.

Nuclear genome differentiation in A-genome diploid species

Based on the results of Southern hybridization analysis, nuclear genomes of 30 accessions in A-genome diploid species were classified (Fig. 25). Most accessions could be individually identified. O. perennis Oceanian form (four accessions in total) was most extremely

differentiated from all others Of this form only one accession was subjected to ctDNA analysis. It had type 9 chloroplast genome, that was exclusively found in O. perennis Oceanian form (Table 7). These results indicate O. perennis Oceanian form is the greatest differentiated group in A-genome diploid species because of its geographical isolation from the rest. Two of the three accessions of O. perennis American form also differentiated greatly from others. Thier chloroplast genome types (types 7 and 8) were unique for this form (Table 7). Therefore, O. perennis American form might have also achieved unique differentiation from other forms and species.

Single accession of both O. perennis American form and African form formed a cluster with O. perennis Asian form and O. sativa ecosp. Indica, and are considered as exceptional types. The accession of O. perennis American form had type 3 chloroplast genome, which was mainly found in O. perennis Asian form and O. sativa ecosp. Indica. As already discussed, this accession is suspected to have been introduced recently to the New Continent. The present results on nuclear genome differentiation also support this assumption. As to the exceptional accession of O. perennis African form, its ctDNA data are not available. However, most of O. perennis African form are perennial and propagate vegetatively, whereas this accession appears annual and multiplies by seeds. Possibly, this accession was originated from other wild species or cultivars by introgression of nuclear genes (Second, personal communication). Analysis of its ctDNA will give some answer to this problem.

As for the rest, wild and cultivated species formed several clusters together as follows: O. perennis Asian form formed a large cluster with O. sativa ecosp. Indica. O. sativa ecosp. Japonica and Javanica composed satellite groups. O. breviligulata and O. glaberrima were included in the same cluster.

Magnitude of nuclear genome differentiation in O. sativa

Based on the results of Southern hybridization analysis, nuclear DNAs of 112 cultivars were classified (Fig. 29). In total, 78 out of 112 cultivars (69.6 %) could be individually identified. Wang and Tanksley (1989) reported that 58 out of 70 cultivars (82.9 %) were differentiated from

one another. The percentages of uniqueness of the nuclear DNA of individual cultivars differed in the two experiments, because the samples used were prepared in different ways as follows: Wang and Tanksley (1989) used five individuals as representative of each cultivar, and examined 50 hybridization patterns (10 probes X 5 endonucleases) for 70 cultivars. In the present experiment, at first a single plant, which had identical isozyme constitution to that reported by Glaszmann (1985), was selected among several plants of each cultivar. Therefore, contaminated seeds or out-fertilized plants, which don't correspond to the accession, could be eliminated.

In scoring of the fragments, only the fragments with strong signal were selected from each hybridization pattern, because it was difficult to judge whether fragments with weak intensity were from the same locus as the probe originated or from different loci of high homology with the probe. In the present study, the number of the fragments scored in each hybridization pattern ranged from zero to three. In most cases, only one fragment was scored, the average fragment number being 1.04 - 1.24 among 112 cultivars. In this way, the magnitude of nuclear genome differentiation revealed by the present analysis will be more or less underestimated than it should be. These are the reasons why the percentage of uniqueness of individual cultivars became lower than that of Wang and Tanksley (1989).

Differentiation at the molecular level of three organellar genomes

The number of observed mutations has been used by many researchers to evaluate the degree of genome differentiation. Especially, chloroplast genome differentiation was clarified in many crop plants, such as Brassica (Palmer et al. 1983), maize (Doebley et al. 1987) and wheat (Ogihara and Tsunewaki 1988). However, it was difficult to apply the same method to mtDNA and nuclear DNA analysis. Alternatively, common restriction fragments observed between two samples were assumed to be homologous DNA sequences, and the rate of fragment changes was used as a parameter of genomic differentiation. This method was successfully applied for the studies of mtDNA and nuclear DNA differentiation (Terachi and Tsunewaki 1986, Song et al. 1988, Wang and Tanksley 1989). In the present investigation, ctDNA variation was evaluated by the number of the

mutations, and those of mtDNA and nuclear DNA were quantified by the percentage of the common fragments.

Based on the results of restriction endonuclease analysis on ct and mtDNA from ten cultivars, the dendrogram showing genetic relationships were constructed (Figs. 7 and 21). As to chloroplast and mitochondrial genome differentiation, most cultivars shared the same chloroplast and mitochondrial genome types, indicating their coevolution through maternal inheritance. By Southern hybridization analysis, their nuclear DNAs were classified into eight types, which were clustered two main groups, O. sativa and O. glaberrima (Fig. 27). These results indicate that nuclear genome differentiation in cultivated species is apparently greater than that of the two organellar genomes.

However, there is one exception to this. One Javanica and one Indica cultivar (Jv2 and I1) did not show remarkable differences in their ctDNAs and nuclear DNAs (100 % and 98 % similarity, respectively), but their mtDNA differed in a great extent (90 % similarity). This is very interesting because Wolfe et al. (1987) reported that the mutation rate of mtDNA is lower than those of ctDNA and nuclear DNA. He compared various nucleotide sequences among ct, mt and nuclear DNA, and concluded that the silent (synonymous) substitution rate in mtDNA is less than one-third of that in ctDNA, or not more than one-fifth of that in nuclear DNA.

Restriction fragment length polymorphisms are assumed to have been caused by mutations. The total number of fragments generated from ct, mt and nuclear DNA of a single cultivar were about 63, 169 and 59, respectively, where all restriction endonucleases used were six-base cutter. Accordingly, the numbers of nucleotides surveyed in ct, mt and nuclear DNA were about 378, 1014 and 354, respectively. Based on the known mutation rates, the nuclear DNA differentiation should be greater than that of mtDNA. However, Wolfe et al. (1987) compared between species, such as wheat and maize, or between monocot and dicot species, which have already developed hybridization barriers and cannot cross with each other. On the other hand, we compared between closely related species or cultivars of the same species. In the case of the Javanica cultivar (J2) and the Indica cultivar (I1), they belong to the same species, O. sativa.

Therefore, one possible reason why their mtDNA showed the largest differentiation among the three kind of DNAs may be the introgression of nuclear DNA. That is, during nuclear genome differentiation, they may have exchanged nuclear DNAs to each other and have consequently had similar nuclear genome constitutions.

Relationships between the chloroplast and nuclear DNA types and Glaszmann's isozyme types in O. sativa

Using the same cultivars as used in the present studies on chloroplast and nuclear genome differentiations in O. sativa (ref. Table 4), Glaszmann (1985, 1987) classified them into six enzymatic groups, i.e., two major (group I and VI), two minor (group II and V) and two satellite groups (group III and IV). The six groups were divided into two primary clusters, one consisting of two subclusters of group I and II, and group III. The other primary cluster contained two subclusters of group V and VI, and group IV. Group I and VI corresponded to typical Indica and Japonica, respectively. Well-known components of group II, III, IV and V are Aus, Deepwater, Rayada and Basmati type cultivars, respectively. Table 27 gives the correspondence between Glaszmann's enzymatic groups and the present chloroplast genome types, and Fig. 32 shows the geographical distribution of all cultivars in relation to their enzymatic and chloroplast genome types. The typical Japonica enzymatic group (group VI) coincides with the Japonica chloroplast genome (type 1), whereas the typical Indica enzymatic group (group I) contains both Japonica (type 1 and 11) and Indica chloroplast genomes (type 3). The near-Indica enzymatic groups (group II and III) also contain both Japonica (type 1) and Indica chloroplast genomes (type 3). As for the near-Japonica enzymatic group, group V has the Japonica chloroplast genomes (type 1 and 12), whereas group IV has the Indica chloroplast genome (type 10). Cultivars from Bangladesh show the widest variation in the combination of different enzymatic and chloroplast genome types.

As to the nuclear genome differentiation in O. sativa, the dendrogram given in Fig. 29 consists of nine major clusters and the number of cultivars with accurate results were scored. In

Table 27. Correspondence between Glaszmann's six enzymatic groups and five chloroplast genome types observed in 68 cultivars of *O. sativa*

Enzymatic group	Ct genome type					Total
	12	11	1	3	10	
I	0	1	5	19	0	25
II	0	0	5	8	0	13
III	0	0	1	1	0	2
IV	0	0	0	0	1	1
V	2	0	8	0	0	10
VI	0	0	13	0	0	13
--	0	0	2	2	0	4
Total	2	1	34	30	1	68

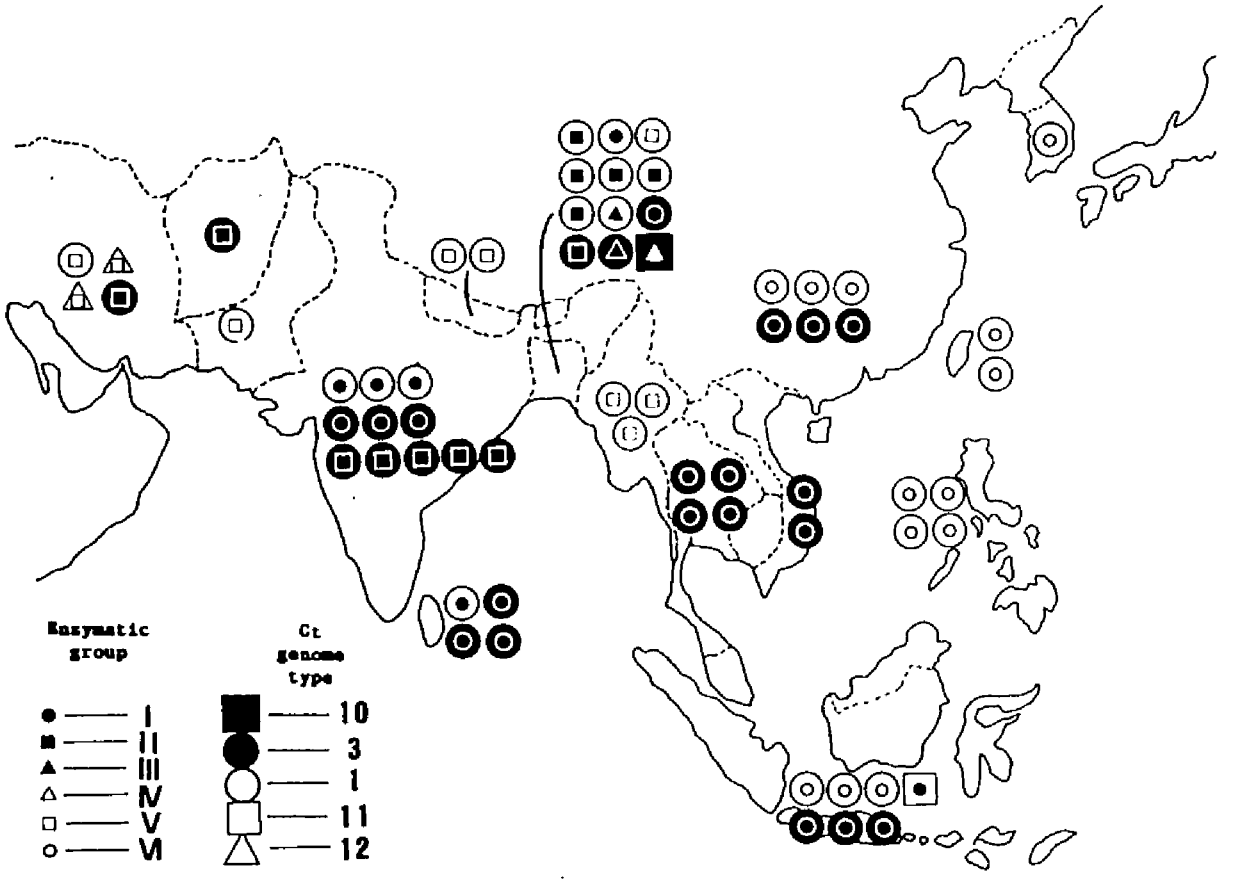


Fig. 32. Geographical distribution of 64 cultivars of O. sativa having different enzymatic (I - VI) and chloroplast genome types (1, 3, 10, 11 and 12).

Fig. 33, the number of cultivars belonging to each cluster with their chloroplast genome types are illustrated. Two of nine clusters consist of the cultivars having type 1 chloroplast genome, whereas most others are a mixture of cultivars with different chloroplast genome types. Similarly, in Fig. 34, the number of cultivars and their enzymatic groups (Glaszmann 1987) are indicated in each of the nine major clusters of nuclear DNAs. Cultivars of enzymatic group VI are found in only two clusters in top, and most cultivars of group I distribute in three clusters in bottom.

Nine major clusters of nuclear DNA types could be divided into three groups, i.e., top one, next five and three bottom clusters. Based on the above results, the first and last groups seem to correspond to typical Japonica and Indica, respectively. Typical Japonica cultivars have only type 1 chloroplast genome and typical Indica cultivars contain mainly type 3 and partly type1 chloroplast genomes.

Phylogenetic relationships between wild and cultivated species

Fig. 25 shows that similar nuclear genomes exist in both wild and cultivated species. In fact, all accessions of O. breviligulata and O. glaberrima belong to one cluster, which share type 5 chloroplast genome in common and exclusively. These facts fully indicate that O. glaberrima originated from O. breviligulata. O. perennis Asian form and O. sativa ecosp. Indica also constitute a cluster, and their magnitudes of the differentiation are almost same. These facts suggest that O. sativa ecosp. Indica with type 1 and type 3 chloroplast genomes originated from O. perennis Asian form having similar nuclear genome constitutions with identical chloroplast genomes, respectively. In the present experiment, no wild accession was found, that has identical or similar nuclear genome to O. sativa ecosp. Japonica and Javanica. This point needs to be investigated in future in order to clarify the origin of O. sativa ecosp. Japonica and Javanica.

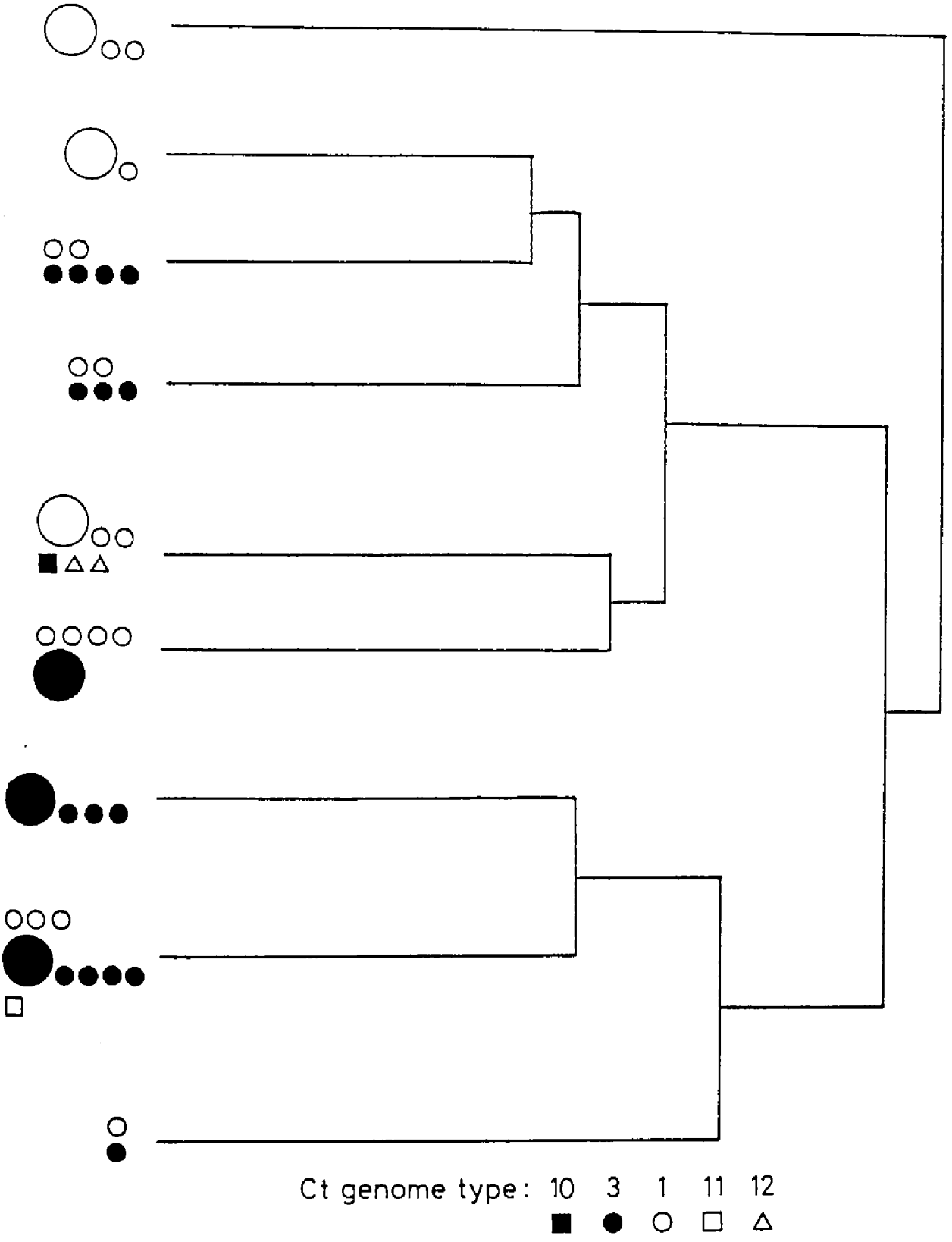


Fig. 33. A Dendrogram showing genetic relationships between nine major clusters of nuclear DNAs with the number of cultivars and their chloroplast genome types belonging to each cluster in O. sativa. Big and small symbols represent five and one cultivars, respectively.

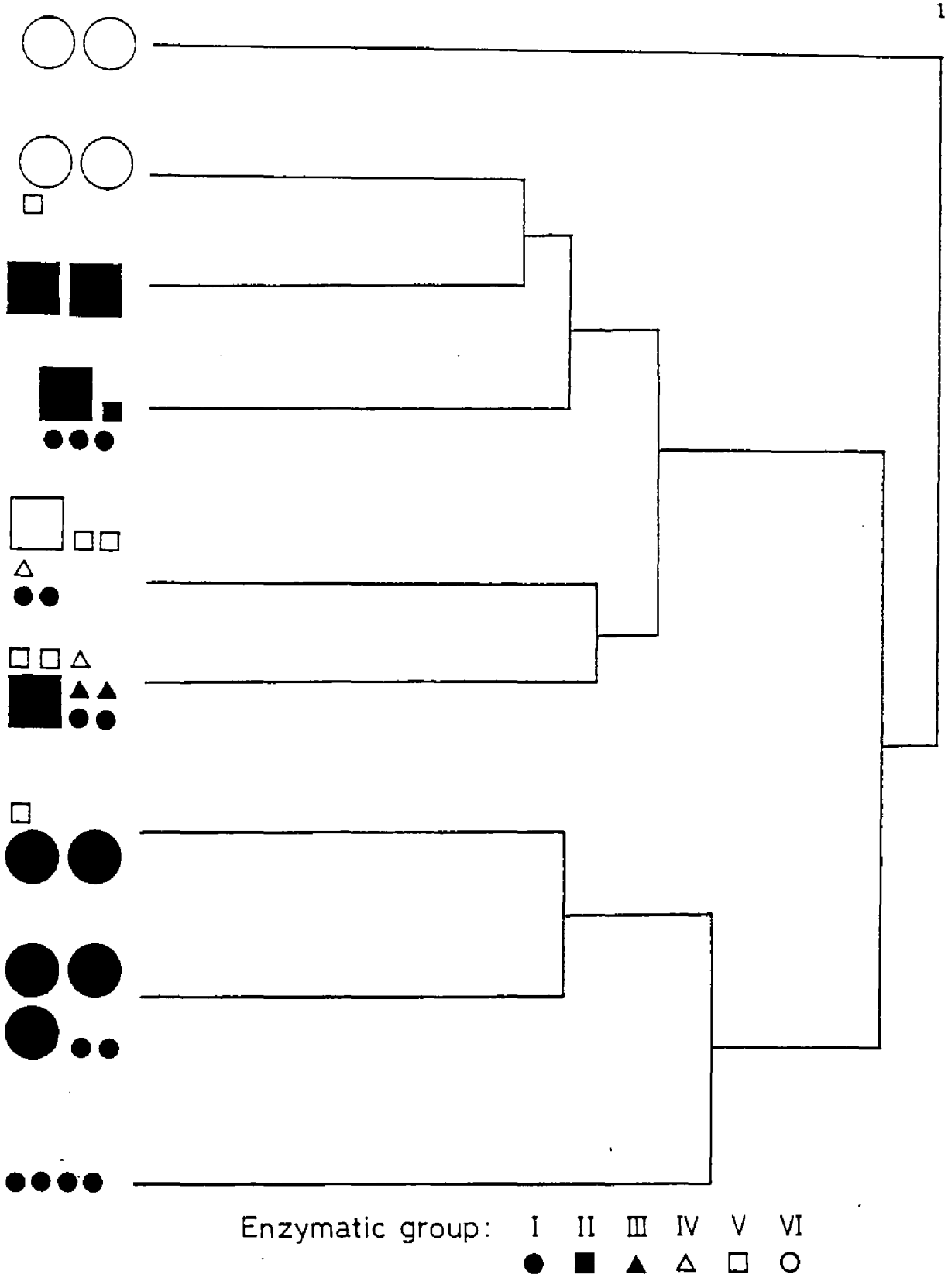


Fig. 34. Dendrogram showing genetic relationships between nine major clusters of nuclear DNAs in O. sativa, with the number of cultivars and their enzymatic groups (Glaszmann 1985), belonging to each cluster. Big and small symbols represent five and one cultivars, respectively.

SUMMARY

In order to elucidate the cytoplasmic and nuclear genome differentiation in A-genome diploid species in the genus Oryza, chloroplast (ct), mitochondrial (mt) and nuclear DNAs among these species were examined by the restriction fragment length polymorphism analysis.

Restiriction endonuclease analysis was carried out to clarify the relationships between chloroplast genomes in A-genome diploid species. Based on the restriction fragment patterns obtained with three endonucleases (EcoRI, HindIII and PstI), chloroplast genomes of 66 accessions in A-genome diploid species (33 O. sativa, 8 O. glaberrima, 21 O. perennis and 4 O. breviligulata accessions) could be classified into nine chloroplast genome types. O. sativa ecosp. Indica and O. perennis Asian and Amirican forms are polymorphic, each possessing three or four chloroplast genome types. In contrast, O. sativa ecosp. Japonica and Javanica, O. glaberrima and O. breviligulata were all found to be monomorphic. Especially, one chloroplast genome type was shared by O. glaberrima and O. breviligulata exclusively, indicating that chloroplast genome of former species had been originated from that of the latter. Two chloroplast genome types were shared by O. sativa and O. perennis Asian form. This suggests that two chloroplast genomes of O. sativa had derived independently from those of O. perennis Asian form.

Detailed studies on three representative chloroplast genomes in cultivated species were carried out by restriction endonuclease analysis with additional eight endonucleases (BamHI, BglII, EcoRV, PvuII, SalI, SmaI, XbaI and XhoI), and by nucleotide sequence analysis of parts of three PstI fragments of ctDNA. The former study revealed new ctDNA variations between the representative chloroplast genomes but not within genomes, indicating strong conservatism of chloroplast genome in cultivated species. The latter detected no difference in 1738 bp among ctDNAs of the representatives. This indicates that nucleotide sequence analysis was applied to relatively conservative regions of ctDNA.

Using 68 local cultivars of O. sativa, ctDNA variation was studied by restriction

endonuclease analysis with six endonucleases (EcoRI, HindIII, PstI, PvuII, SmaI and XhoI). Based on the restriction fragment patterns, they could be classified into five types. Among them, four length mutations and two base substitutions were found and their locations were identified on the PstI physical map of ctDNA. By cluster analysis, five chloroplast genomes are mainly divided into two groups, namely, Japonica and Indica chloroplast genomes. Both groups are distributed widely in Asian countries. The Japonica enzymatic group classified by Glaszmann (1985) carries only the Japonica chloroplast genome, whereas the Indica enzymatic group contains both Japonica and Indica chloroplast genomes.

MtDNA variation among ten cultivars (8 O. sativa and 2 O. glaberrima cultivars) were surveyed by restriction endonuclease and Southern hybridization analyses. Their mtDNAs were digested with five endonucleases (BamHI, HindIII, PstI, PvuII and XhoI), and hybridization was performed with four mtDNA probes (ATPA and COXII from pea, and RRN18&5 and RRN26 from wheat). In both analyses, ten cultivars could be divided into four groups. As to the relationships between their chloroplast and mitochondrial genome differentiation, most cultivars shared the same chloroplast and mitochondrial genome types. This coincidence seems to reflect their coevolution through maternal inheritance, and indicates conservatism of mitochondrial genome in cultivated species.

Southern hybridization analysis with 12 single-copy rice nuclear DNA probes were carried out. First, nuclear DNAs of 30 accessions in A-genome diploid species (8 O. sativa, 6 O. glaberrima, 13 O. perennis and 3 O. breviligulata accessions) were digested with two endonucleases (HindIII and PstI). Restriction fragment length polymorphisms of their nuclear DNAs were examined by cluster analysis. O. perennis Oceanian and American forms differentiated greatly from others, whereas O. perennis Asian form and O. breviligulata formed clusters with O. sativa ecosp. Indica and O. glaberrima, respectively. This indicates nuclear genomes of O. sativa ecosp. Indica and O. glaberrima had originated from those of O. perennis Asian form and O. breviligulata, respectively. Secondly, 112 local cultivars of O. sativa were analyzed by the same method as described above. In total, 78 out of 112 cultivars were individually identified, and their

nuclear genomes could be classified into nine major groups. As compared with their chloroplast genome types and isozyme constitutions (Glaszmann 1985), one and three groups out of nine seem to correspond to typical Japonica and Indica cultivars, respectively.

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