

CYTOPLASMIC AND NUCLEAR GENOME DIFFERENTIATION IN A-GENOME DIPLOID

SPECIES OF RICE AS REVEALED BY THE RESTRICTION FRAGMENT LENGTH

POLYMORPHISM ANALYSIS OF DNAS

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1991

CONTENTS

I. INTRODUCTION	1
II. MATERIALS AND METHODS	
A. Materials	6
B. Methods	
CtDNA extraction	14
MtDNA extraction	15
Nuclear DNA extraction	15
Total DNA extraction	15
Restriction endonuclease analysis	16
Cloning ctDNA fragments	16
Cloning nuclear DNA fragments	17
Probe DNA preparation	17
Southern hybridization with radioactive probes ...	18
Southern hybridization with non-radioactive probes	18
Sequence analysis	19
Isozyme analysis	19
III. RESULTS	
A. Chloroplast genome differentiation in A-genome diploid species	
Restriction endonuclease analysis on ctDNA variation in four A-genome diploid species	21
Detailed studies on ctDNA variation in <u>O. sativa</u> and <u>O. glaberrima</u>	21
Molecular differences between type 1, 3, 4 and 5 chloroplast genomes revealed by Southern hybridization	29

Nucleotide sequence analysis on ctDNA from type 1, 3 and 5 chloroplast genomes	34
CtDNA variation in <u>O. sativa</u>	39
B. Mitochondrial genome differentiation between <u>O. sativa</u> and <u>O. glaberrima</u>	
Restriction endonuclease analysis of mtDNA	59
Southern hybridization analysis of mtDNA	59
C. Nuclear genome differentiation in A-genome diploid species	
Nuclear genome differentiation in A-genome diploid species	68
Nuclear genome differentiation between <u>O. sativa</u> and <u>O. glaberrima</u>	68
Nuclear genome differentiation in <u>O. sativa</u>	74
IV. DISCUSSION	
Chloroplast genome differentiation in wild A-genome species	86
Chloroplast genome relationships between wild and cultivated species	87
Molecular differences between three major chloroplast genomes found in cultivated species ..	89
Chloroplast genome differentiation in <u>O. sativa</u> ..	91
Geographical distribution of different chloroplast genomes in <u>O. sativa</u>	93
Mitochondrial genome differentiation in cultivated species, and comparison between mitochondrial and chloroplast genome differentiation	93
Nuclear genome differentiation in A-genome diploid species	94
Magnitude of nuclear genome differentiation in <u>O. sativa</u>	95
Differentiation at the molecular level of three organellar genomes	96

Relationships between the chloroplast and nuclear DNA types and Glaszmann's isozyme types in <u>O. sativa</u>	98
Phylogenetic relationships between wild and cultivated species	101
SUMMARY	104
ACKNOWLEDGEMENTS	107
REFERENCES	109

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 species 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). Conceptual 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. Biochemical 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

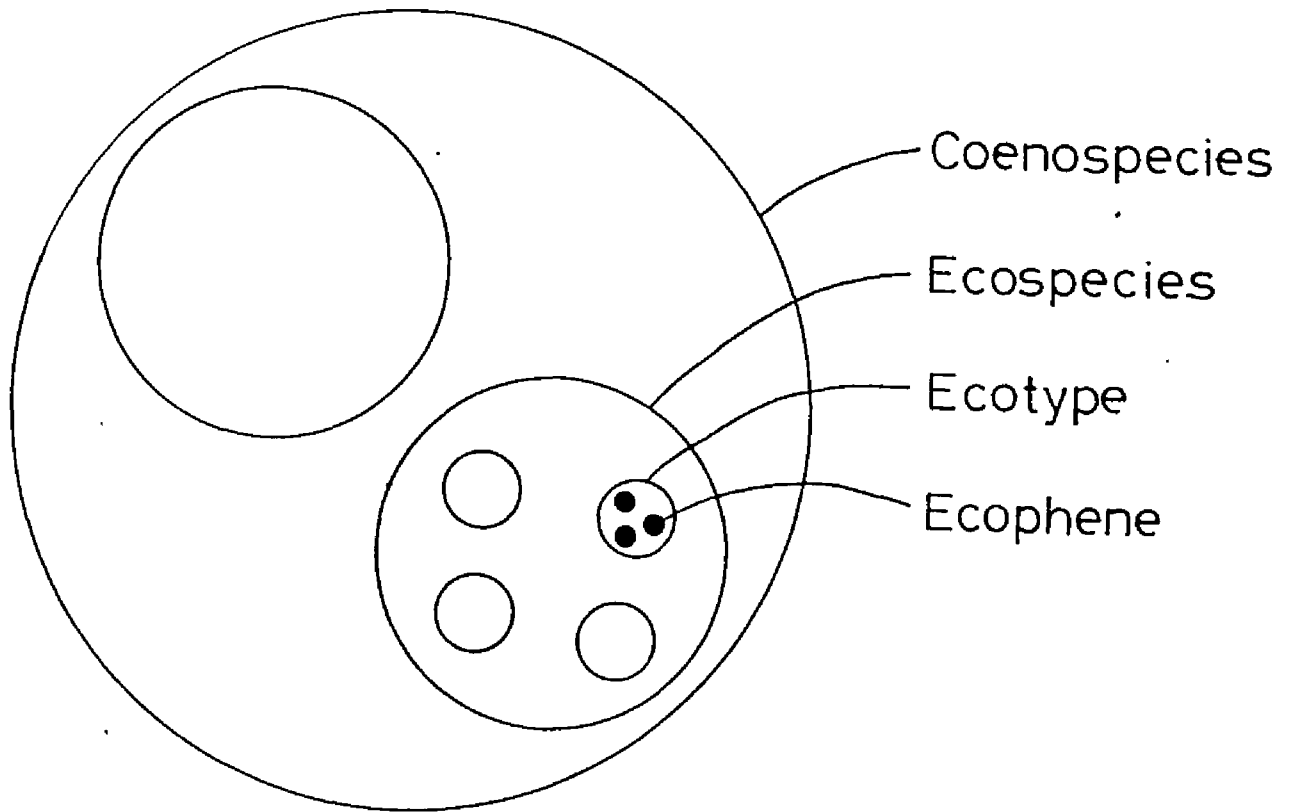


Fig. 1. Turesson's scheme showing the relationships among coenospecies, ecospecies, ecotype and ecophene.

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; Ogiwara 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 Dally 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⁾
<u>O. sativa</u>	ecosp. <u>Japonica</u>	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
<u>O. sativa</u>	ecosp. <u>Javanica</u>	242	S	Philippines
"	"	532	S	Japan
"	"	647	S	Philippines
"	"	(C5810) Taichung No. 65 ³⁾	S	Indonesia
"	"	(C5811) Taichung No. 65 ³⁾	S	Indonesia
<u>O. sativa</u>	ecosp. <u>Indica</u>	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	---
<u>O. glaberrima</u>		(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⁾
<u>O. perennis</u>	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
<u>O. breviligulata</u>		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	<u>O. sativa</u> ecosp. <u>Japonica</u>	Nipponbare
J 2	" "	Taichung No. 65
J 3	" "	Norin No. 22
J 4	" "	Aichiasahi
J 5	" "	501
Jv1	" ecosp. <u>Javanica</u>	532
Jv2	" "	647
Jv3	" "	(C5810) Taichung No. 65 ¹⁾
Jv4	" "	(C5811) Taichung No. 65 ¹⁾
I 1	" ecosp. <u>Indica</u>	419
I 2	" "	C8005
I 3	" "	108
I 4	" "	IR36
I 5	" "	104
I 6	" "	C5444
G 1	<u>O. glaberrima</u>	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	<u>O. perennis</u> 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	<u>O. breviligulata</u>	W607
15	"	W653
16	"	W1152
17	<u>O. sativa</u> ecosp. <u>Indica</u>	IR36
18	" ecosp. <u>Japonica</u>	Nipponbare
19	" "	Norin No.22
20	" ecosp. <u>Javanica</u>	532
21	" "	647
22	" ecosp. <u>Indica</u>	108
23	" "	419
24	" "	C8005
25	<u>O. glaberrima</u>	W401
26	"	W438
27	"	W440
28	"	W446
29	"	W492
30	"	W528

B. Methods

CtDNA extraction

CtDNA extraction was carried out after Tsunewaki and Ogiwara (1983) and Enomoto *et al.* (1985). About 50 gm of seedling leaves were cut into 1 cm long, and homogenized in liquid nitrogen twice, each for 6 sec at 18,000 rpm using a homogenizer (Nissei AM-7, Nihonseiki Co. Ltd., Japan). After liquid nitrogen 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 °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 MgCl₂; 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 nucleus 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 temperature 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 Frozen 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 °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. Cloning 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 DH5 α 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 °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 °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 °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 °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 MgCl₂; 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 staining 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

Restriction 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 American 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

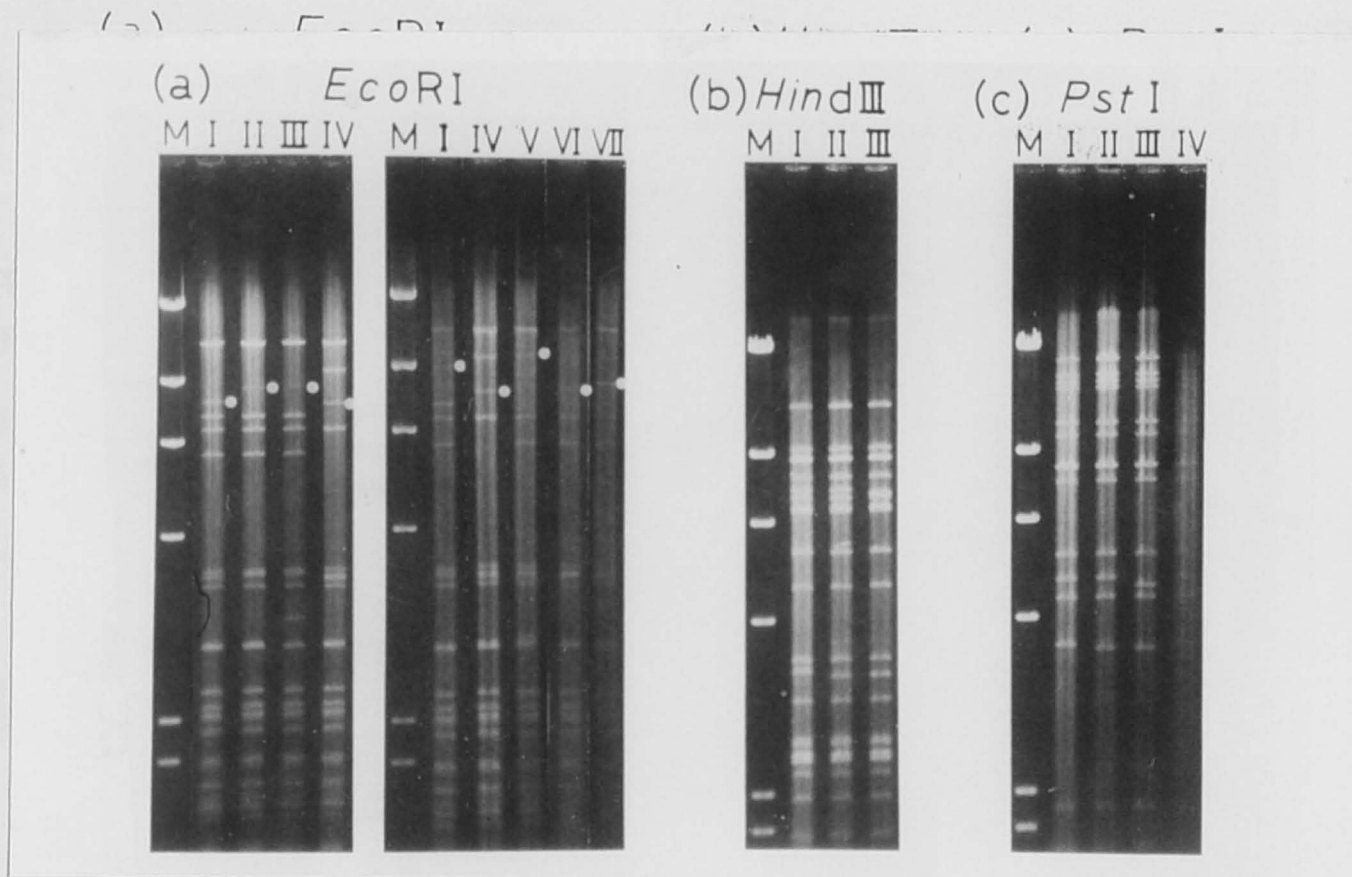


Fig. 2. *EcoRI*, *HindIII* and *PstI* restriction fragment patterns of ctDNA found in four A-genome diploid species. In *EcoRI* restriction fragment patterns, rDNA fragments (marked with the circle) which are contamination of nuclear DNA to ctDNA sample are found.

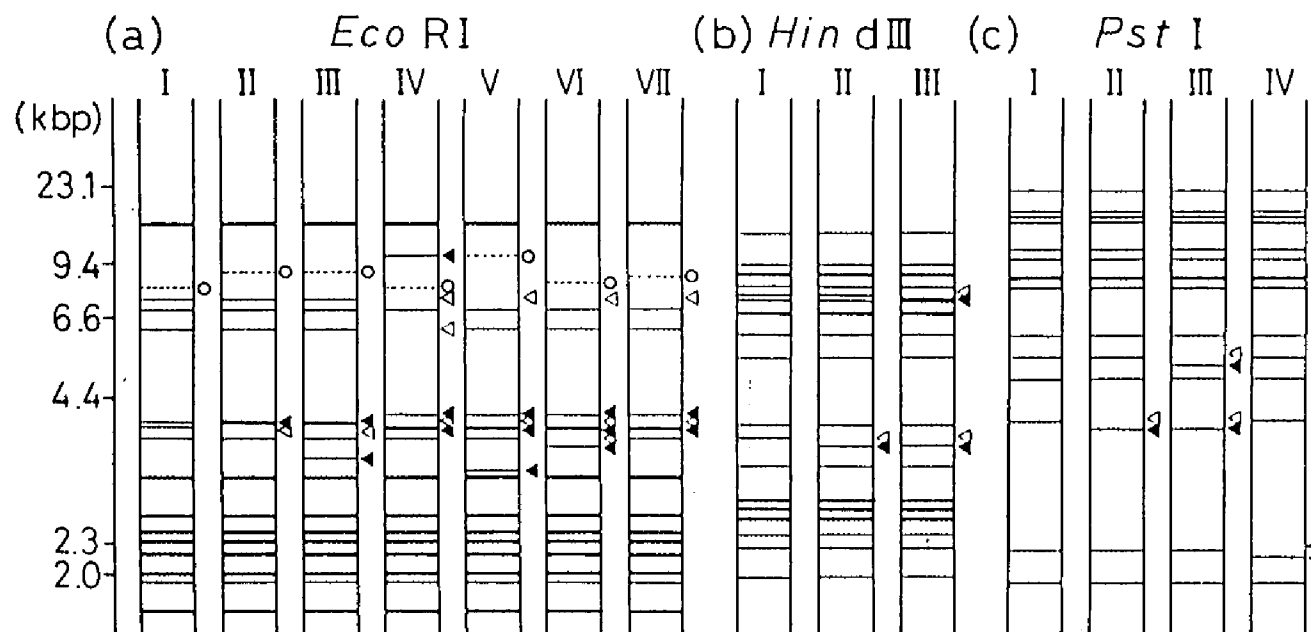


Fig. 3. Schematic representation of seven EcoRI (a), three HindIII (b) and four PstI (c) restriction fragment patterns of ctDNA found among 66 accessions of four A-genome diploid species.

◁ 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

<u>EcoRI</u>								
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)

<u>HindIII</u>					<u>PstI</u>					
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		
		<u>EcoRI</u>	<u>HindIII</u>	<u>PstI</u>
<u>O. sativa Japonica</u>	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
<u>O. sativa Javanica</u>	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
<u>O. sativa Indica</u>	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
<u>O. glaberrima</u>	(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		
		<u>EcoRI</u>	<u>HindIII</u>	<u>PstI</u>
<u>Q. perennis</u>	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
<u>Q. breviligulata</u>	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	
<u>EcoRI</u> frag. pattern	I	I	II	III	IV	V	VI	VI	VII	
<u>HindIII</u> frag. pattern	I	II	II	III	I	I	I	I	I	
<u>PstI</u> frag. pattern	I	II	II	III	I	I	I	IV	I	
<u>O. sativa</u> Japonica	11	--	--	--	--	--	--	--	--	11
" Javanica	5	--	--	--	--	--	--	--	--	5
" Indica	3	--	13	1	--	--	--	--	--	17
<u>O. glaberrima</u>	--	--	--	--	8	--	--	--	--	8
<u>O. perennis</u> Asian	3	1	5	--	--	--	--	--	--	9
" American	--	--	1	--	--	2	1	1	--	5
" African	--	--	--	--	--	5	--	--	--	5
" Oceanian	--	--	--	--	--	--	--	--	2	2
<u>O. breviligulata</u>	--	--	--	--	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 Q. 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 distinguished from the former by BglII, EcoRI, HindIII, PstI and XbaI fragment patterns. The third type was found in all accessions of Q. 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.

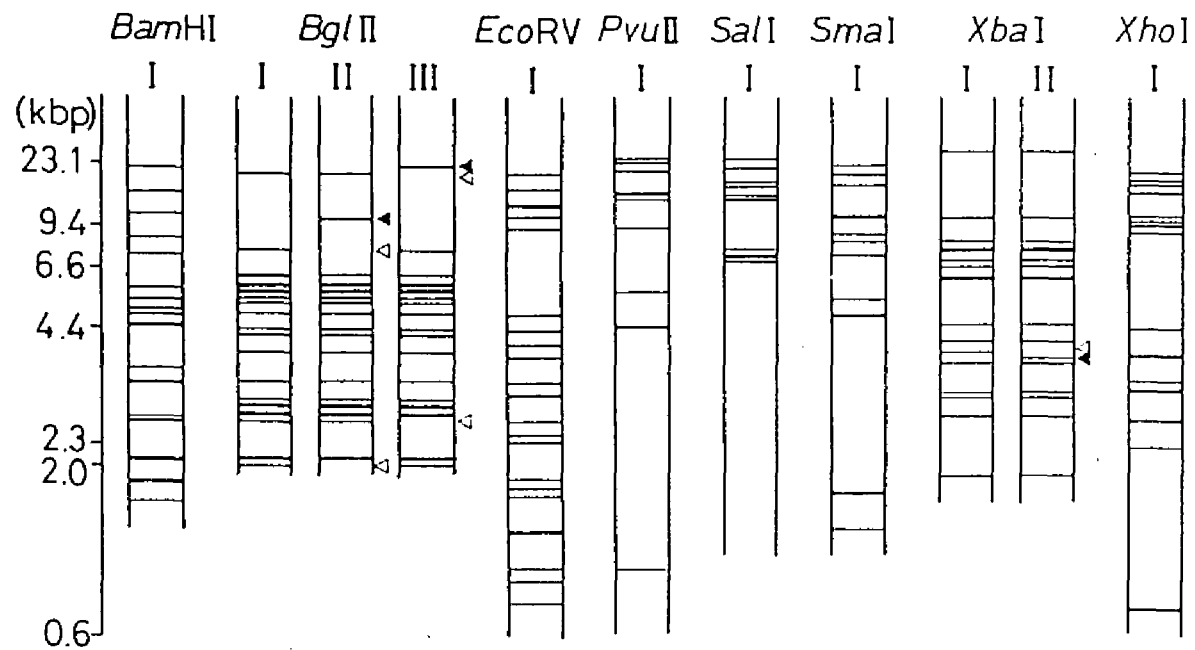


Fig. 4. Schematic representation of *Bam*HI, *Bgl*II, *Eco*RV, *Pvu*II, *Sal*I, *Sma*I, *Xba*I and *Xho*I restriction fragment patterns of ctDNA found among 19 accessions of two cultivated species.

◁ 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

<u>Bam</u> HI			<u>Bgl</u> II					<u>Eco</u> RV		
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)

<u>PvuII</u>			<u>SalI</u>			<u>SmaI</u>		
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	+

<u>XbaI</u>				<u>XhoI</u>		
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⁾
	<u>Bam</u> HI	<u>Bgl</u> II	<u>Eco</u> RV	<u>Pvu</u> II	<u>Sal</u> I	<u>Sma</u> I	<u>Xba</u> I	<u>Xho</u> I	<u>Eco</u> RI	<u>Hin</u> dIII	<u>Pst</u> 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 genomes

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

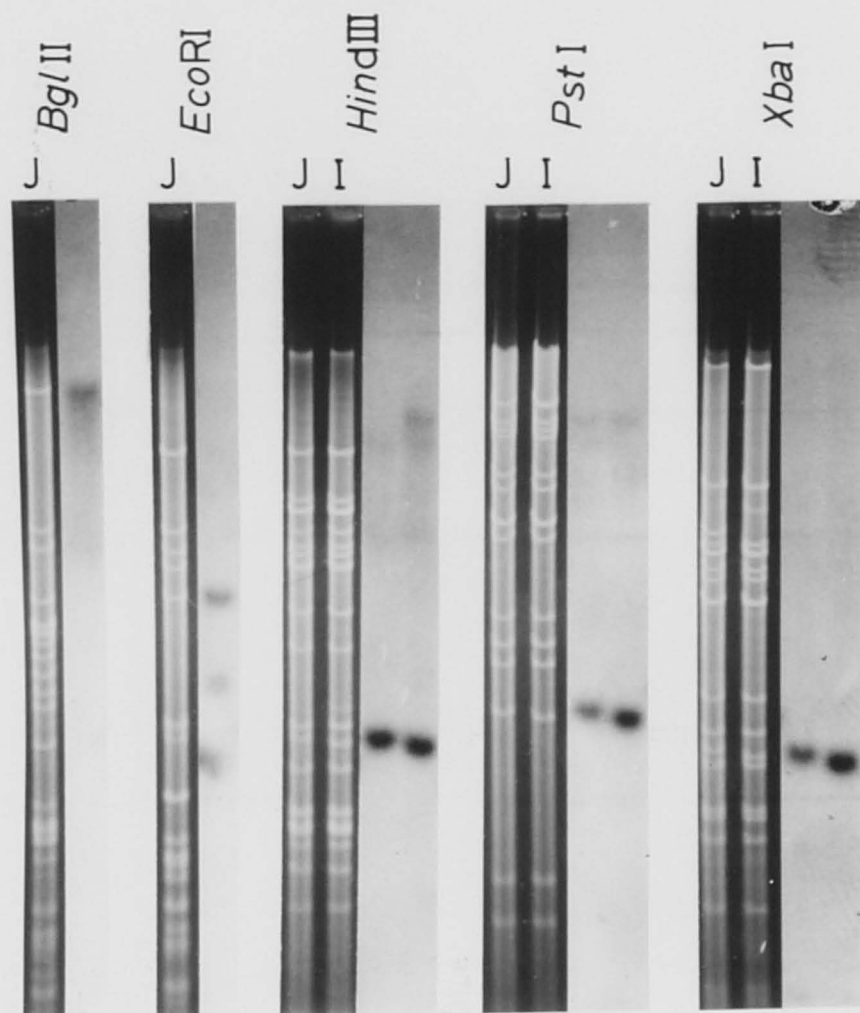


Fig. 5. Southern hybridization patterns of the P12 fragment as probe to *Bgl*II, *Eco*RI, *Hind*III, *Pst*I and *Xba*I digests of *O. sativa* ecosp. *Japonica* cv. Nipponbare ctDNA (J), and to *Hind*III, *Pst*I and *Xba*I digests of ecosp. *Indica* cv. IR36 (I).

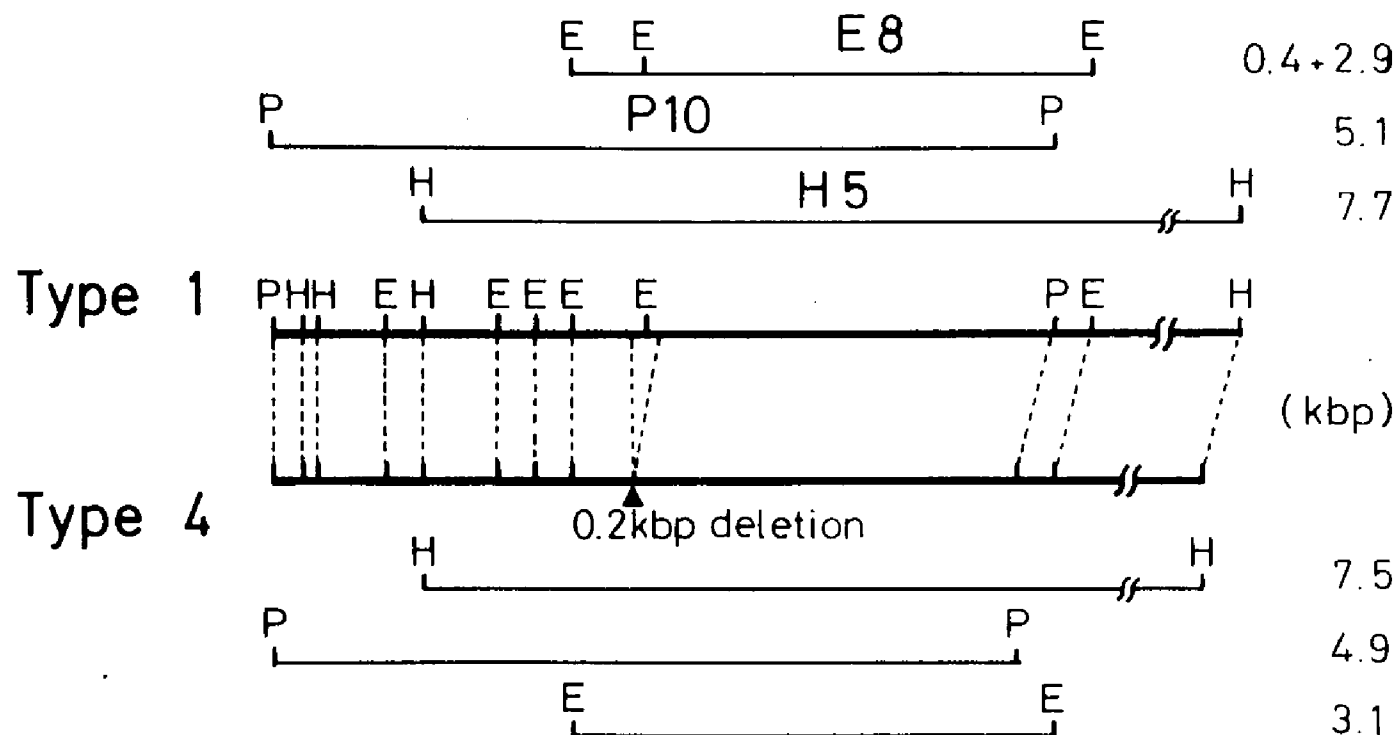


Fig. 6. Physical maps of the P10 fragments of type 1 and 4 chloroplast genomes, confirming the size of the fragments generated by EcoRI, HindIII and PstI-digestion.

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 (<u>EcoRI</u>)	1 vs 5	7.4 (E2) vs 3.7 + 3.7
Site loss (<u>EcoRI</u>)	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				--

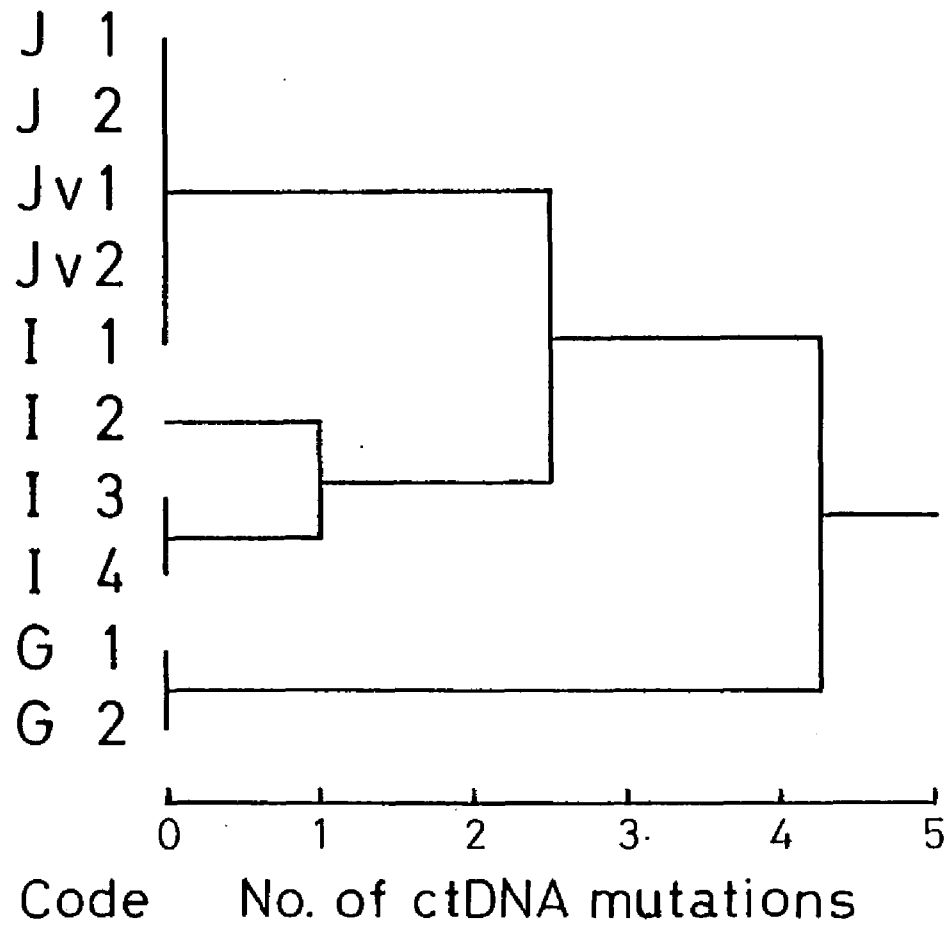


Fig. 7. Dendrogram showing genetic relationships between chloroplast genomes of ten cultivars of *Oryza* based on the number of ctDNA mutations.

cv. IR36, and *O. glaberrima* strain W401. In order to clarify the differences between them at DNA sequence level, three *Pst*I 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 fragments.

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 CF₀ (*atpH*) (Rodermeier 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 CF₁ (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.

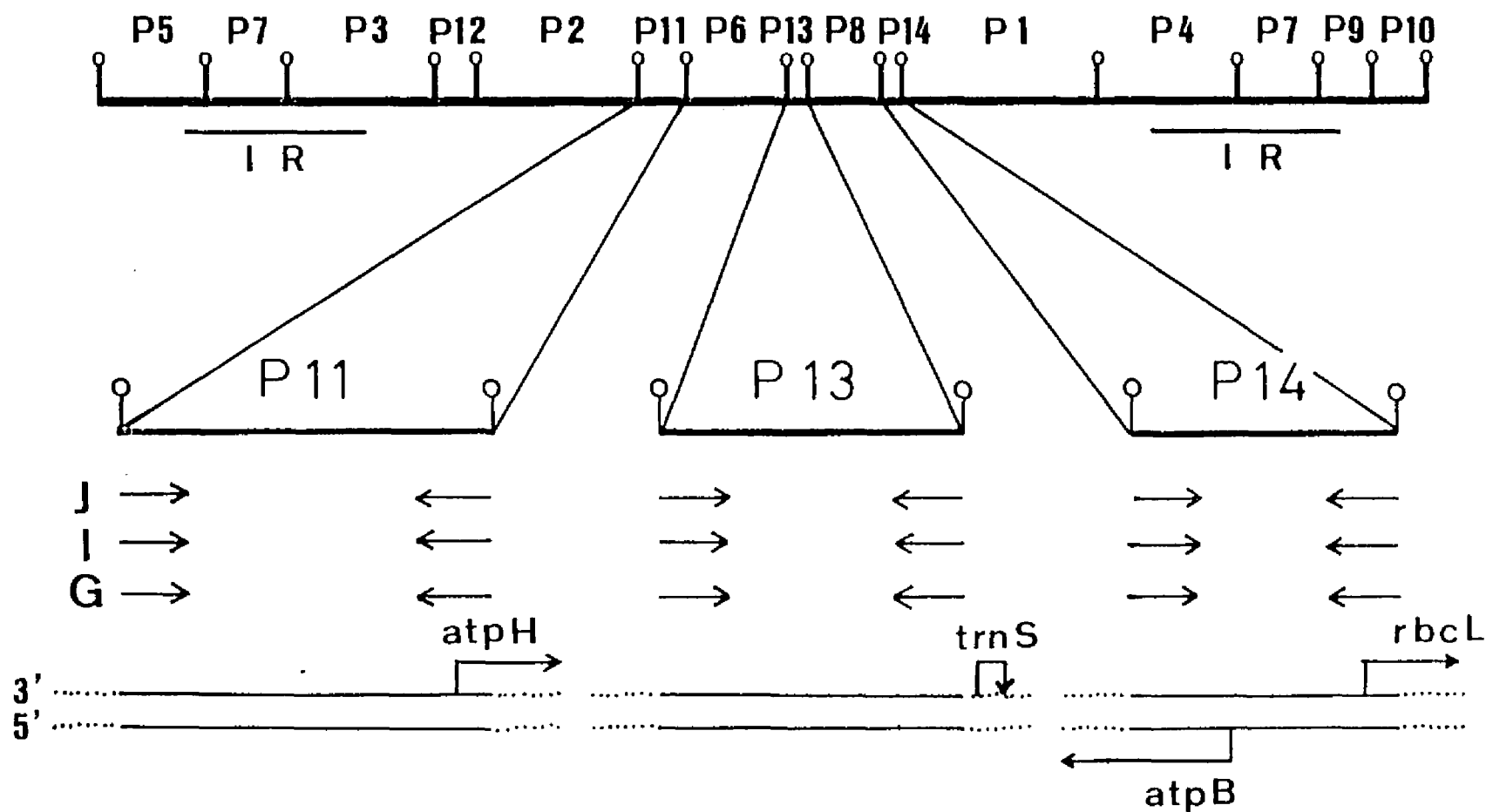


Fig. 8. Locations of three *Pst*I fragments, P11, P13 and P14, and the strategies employed in their nucleotide sequencing.

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	TCCGAAGTGC	TTCGAGATCT
250	260	270	280	290	300
CCTTTTTAGT	TTCTAATCAT	TAGAGGTTTG	TGTACTCATT	ATTCTATTTT	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	AACTTTTCC	CACAGGATCT
70	80	90	100	110	120
CTTGCAGGAA	GAGGGTAATC	TCCAAGTTCG	ACTTGTCAT	TTTATTTCTC	ATGAAAATAG
130	140	150	160	170	180
CAAGTTAACT	CAAAGAATTT	ATCATACGAA	TAGTCAATTC	GTTGGAAGTT	GCTTAGTATG
190	200	210	220	230	240
AATTGGGAAC	AAGAAGAAAA	AGAGGAGGCT	CGTGCTTCCC	TTGTTGAGAT	AAGAGCAAAT
250	260	270	280	290	300
GGTCTGATTC	GCGATTTTCT	AAGAATTGGG	TTAATCAAAT	CCACTATTTT	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 AAAGTTTGTAG 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 CGGTTTCATTC ATTTGGCCAT AGACTAGAGC TACCTTTGAT

     210     220     230     240     250
TCCTCAAGAT TTTTTTCATT AATTACTCCA GATTCCTTCA TTTCCATATA

     260     270     280
AAGATCATTT CCTTCACGAG TCCGTTCCC

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      10      20      30      40      50
CTGCAGCCCC TGCTTCTTCG GGC GGAACCC 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

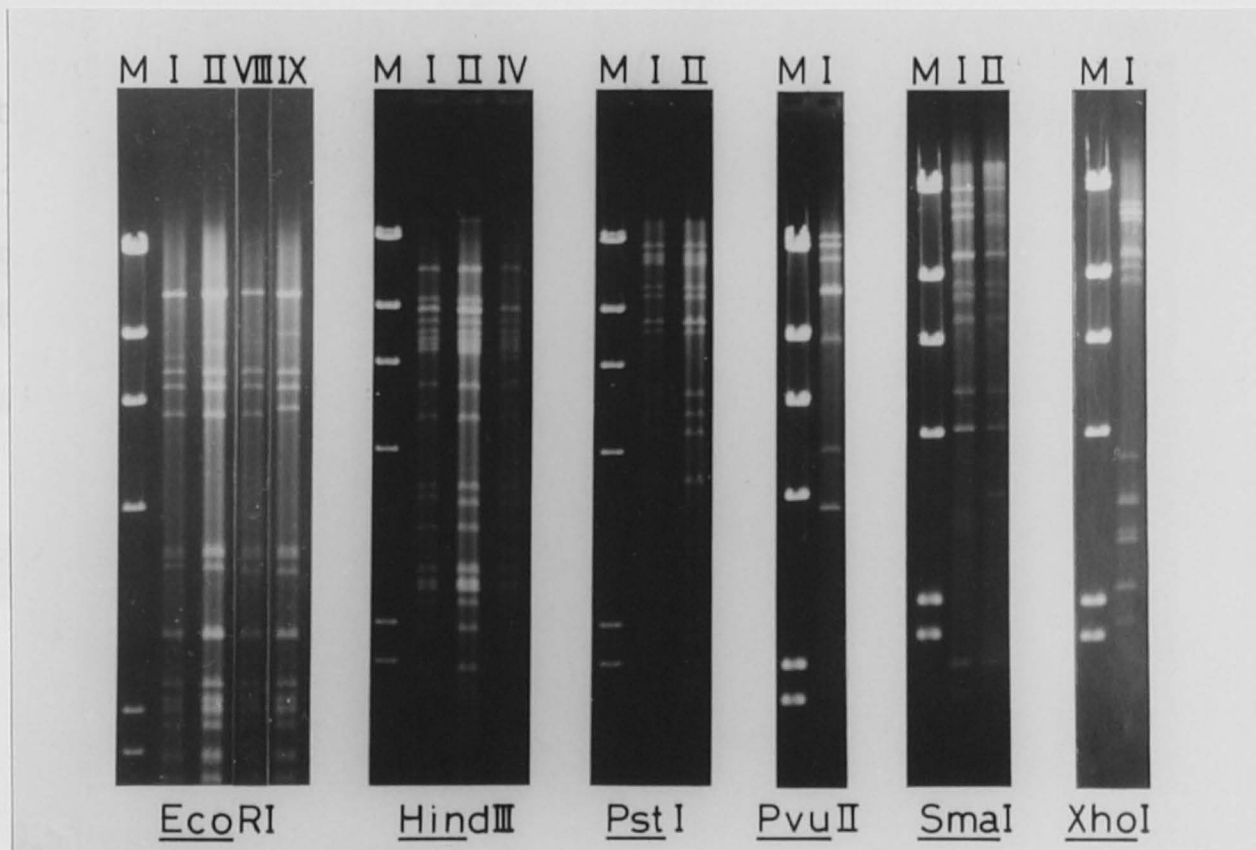


Fig. 12. EcoRI, HindIII, PstI, PvuII, SmaI and XhoI restriction fragment patterns of ctDNA observed among 68 Asian cultivars of *O. sativa*.

M: Lambda DNA digested with HindIII as molecular markers.

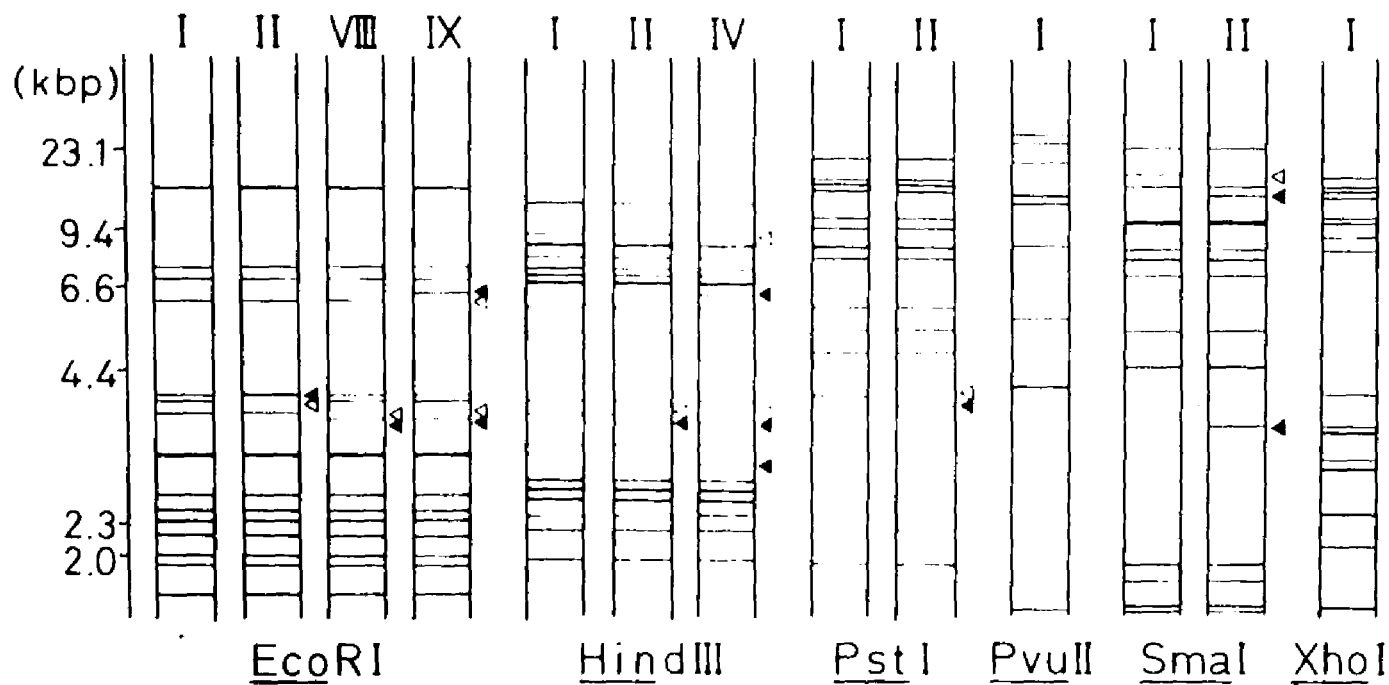


Fig. 13. Schematic representation of EcoRI, HindIII, PstI, PvuII, SmaI and XhoI restriction fragment patterns of ctDNA observed among 68 Asian cultivars of O. sativa.

◁ 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*

<u>EcoRI</u>						<u>HindIII</u>					<u>PvuII</u>		
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)

<u>PstI</u>				<u>SmaI</u>				<u>XhoI</u>		
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 patterns. Materials used as the source of chloroplast and nuclear DNA

No.	Acc.No	Rest. frag. Pattern						Ct genome type
		<u>EcoRI</u>	<u>HindIII</u>	<u>PstI</u>	<u>PvuII</u>	<u>SmaI</u>	<u>XhoI</u>	
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
		<u>EcoRI</u>	<u>HindIII</u>	<u>PstI</u>	<u>PvuII</u>	<u>SmaI</u>	<u>XhoI</u>	
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
	<u>EcoRI</u>	<u>HindIII</u>	<u>PstI</u>	<u>PvuII</u>	<u>SmaI</u>	<u>XhoI</u>	
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	<u>EcoRI</u>	E6	3.7 vs 3.8
"	<u>HindIII</u>	H12	3.6 vs 3.5
"	<u>PstI</u>	P12	3.9 vs 3.8
1 vs 10	<u>EcoRI</u>	E6	3.7 vs 3.8
"	<u>HindIII</u>	H2	9.6 vs 6.6 + 3.0
"	<u>HindIII</u>	H12	3.6 vs 3.5
"	<u>PstI</u>	P12	3.9 vs 3.8
"	<u>SmaI</u>	Sm2	17.4 vs 14.0 + 3.4
1 vs 11	<u>EcoRI</u>	E7	3.6 vs 3.5
1 vs 12	<u>EcoRI</u>	E4	6.1 vs 6.4
"	<u>EcoRI</u>	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)				(b)	
Probe (kbp)	Ct genome type	Enzyme	Hybridized fragment (kbp)	Probe	<u>Pst</u> I hybridized fragment
E4 (6.1)	12	<u>Eco</u> RI	6.4	E4	P2, P12
E6 (3.7)	3, 10	"	3.8	E6	P2
E7 (3.6)	11, 12	"	3.5	E7	P1
H2 (9.6)	10	<u>Hind</u> III	6.6 + 3.0	H2	P1, P8, P14
Sm2 (17.4)	10	<u>Sma</u> I	14.0 + 3.4	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).

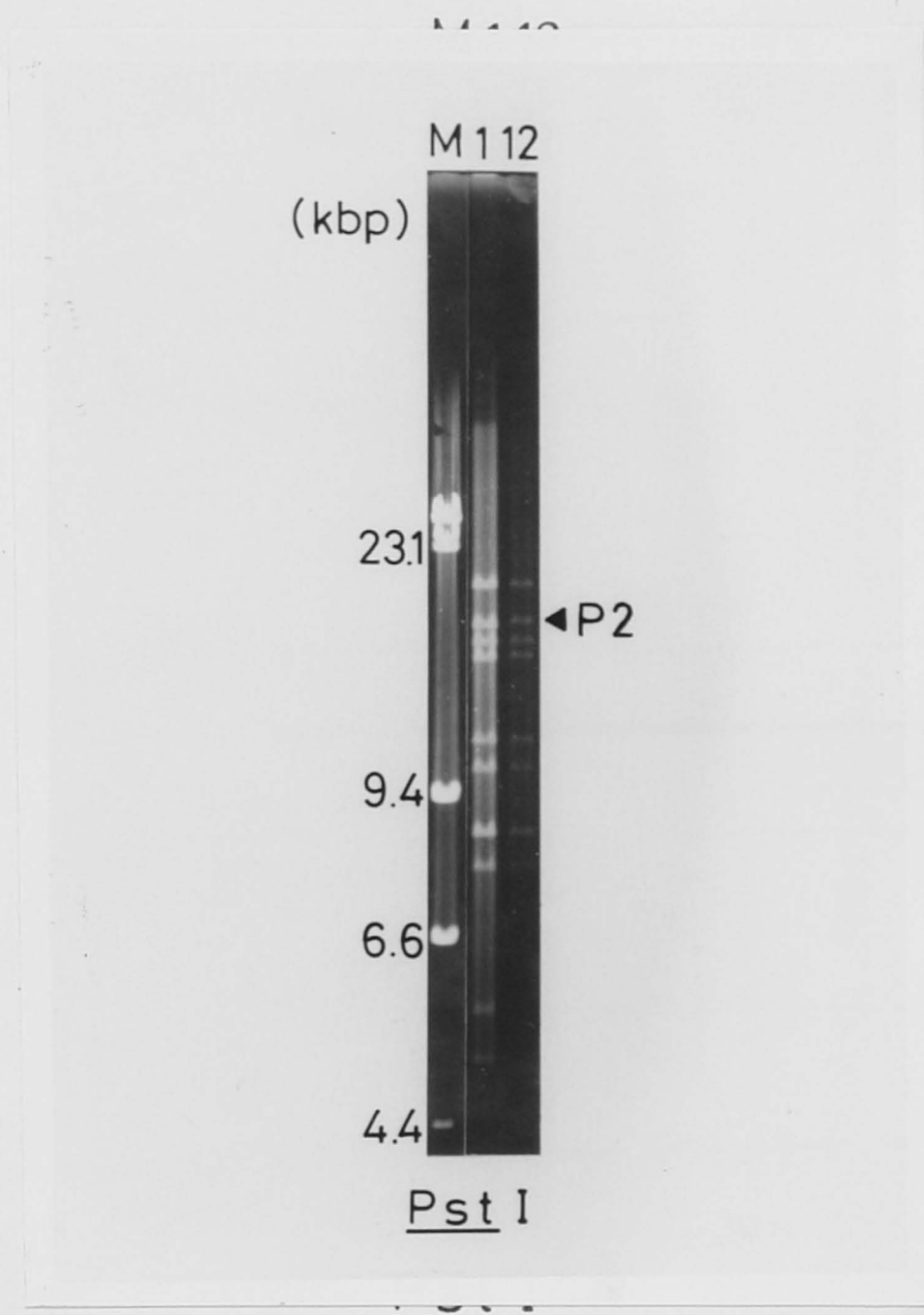


Fig. 14. PstI restriction fragment patterns of ctDNA from the type 1 and 12 chloroplast genomes using 0.5% agarose gel.

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

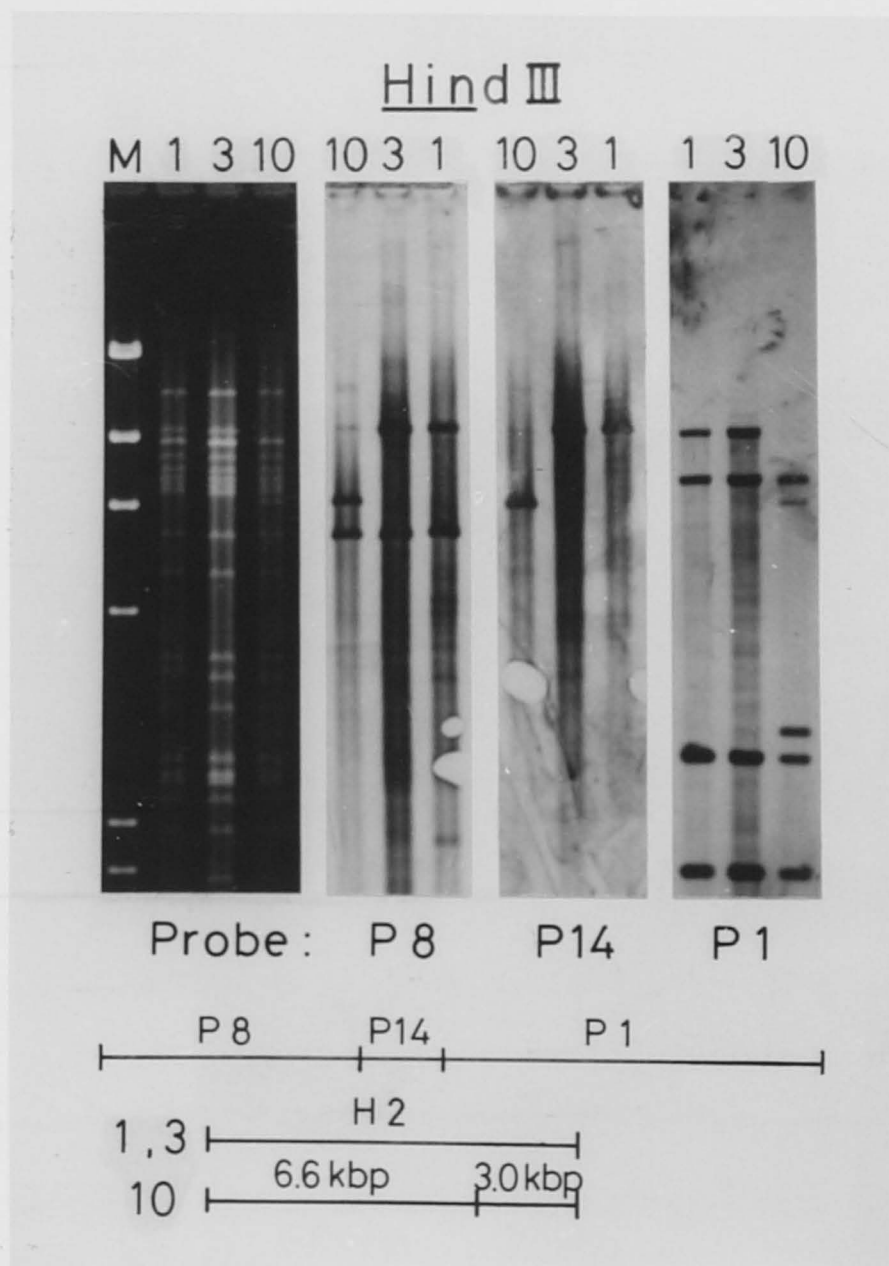


Fig. 15. Hybridization patterns of the P8, P14 and P1 fragments as probes to the HindIII fragments from the type 1, 3 and 10 chloroplast genomes, confirming the location of the site gain in the H2 fragment and the corresponding region of the PstI physical map.

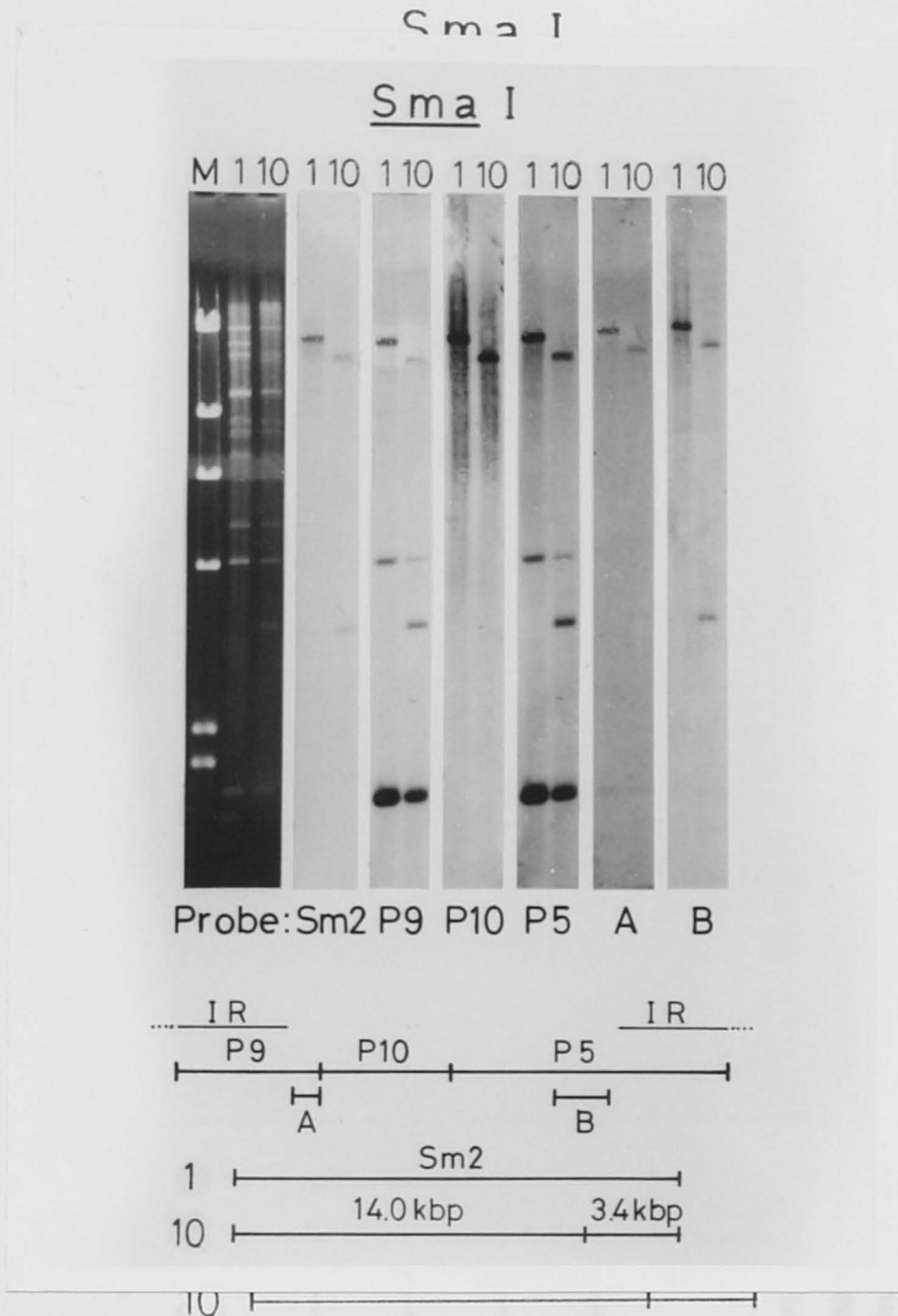


Fig. 16. Hybridization patterns of the Sm2, P9, P10, P5, A and B fragments as probes to the SmaI fragments from the type 1 and 10 chloroplast genomes, confirming the location of the site gain in the Sm2 fragment and the corresponding region of the PstII physical map. A and B are a 0.89 kbp (EcoRI-PstII) 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.

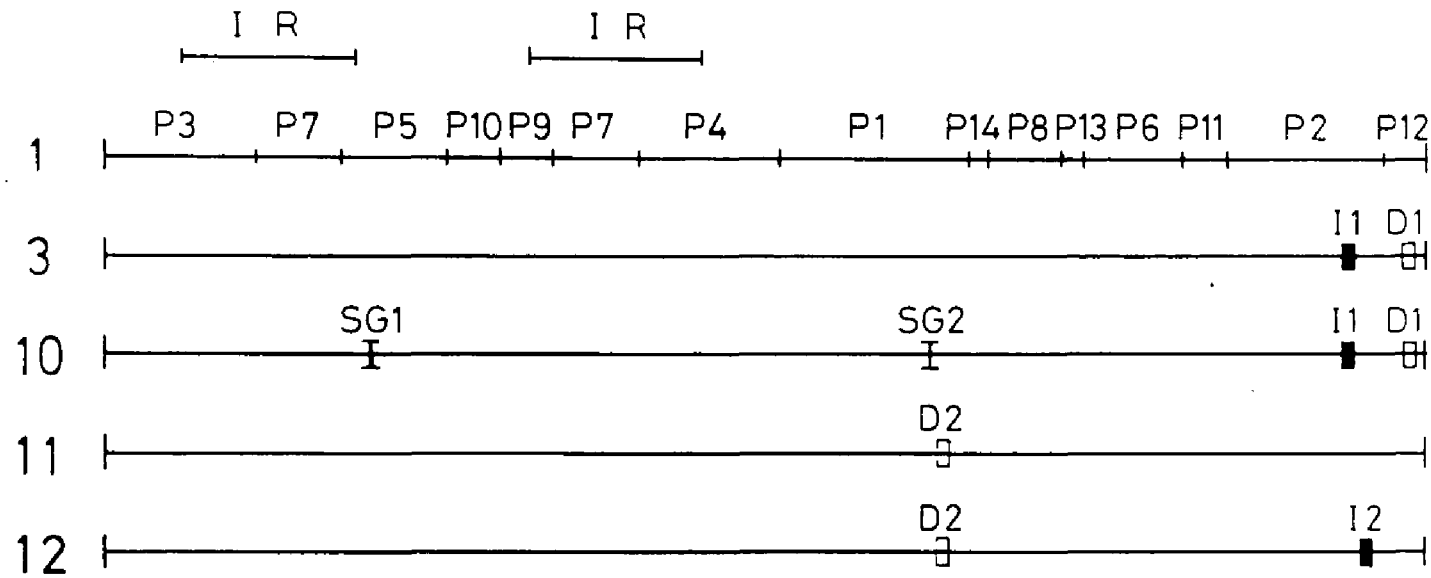


Fig. 17. Physical map differences of five chloroplast genome types found among 68 cultivars of *O. sativa*. On the top, *Pst*I physical map in the type 1 chloroplast genome is shown. Mutations are indicated in the maps of the type 3, 10, 11 and 12 chloroplast genome as compared with the physical map 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, *Sma*I site gain and *Hind*III site gain, respectively.

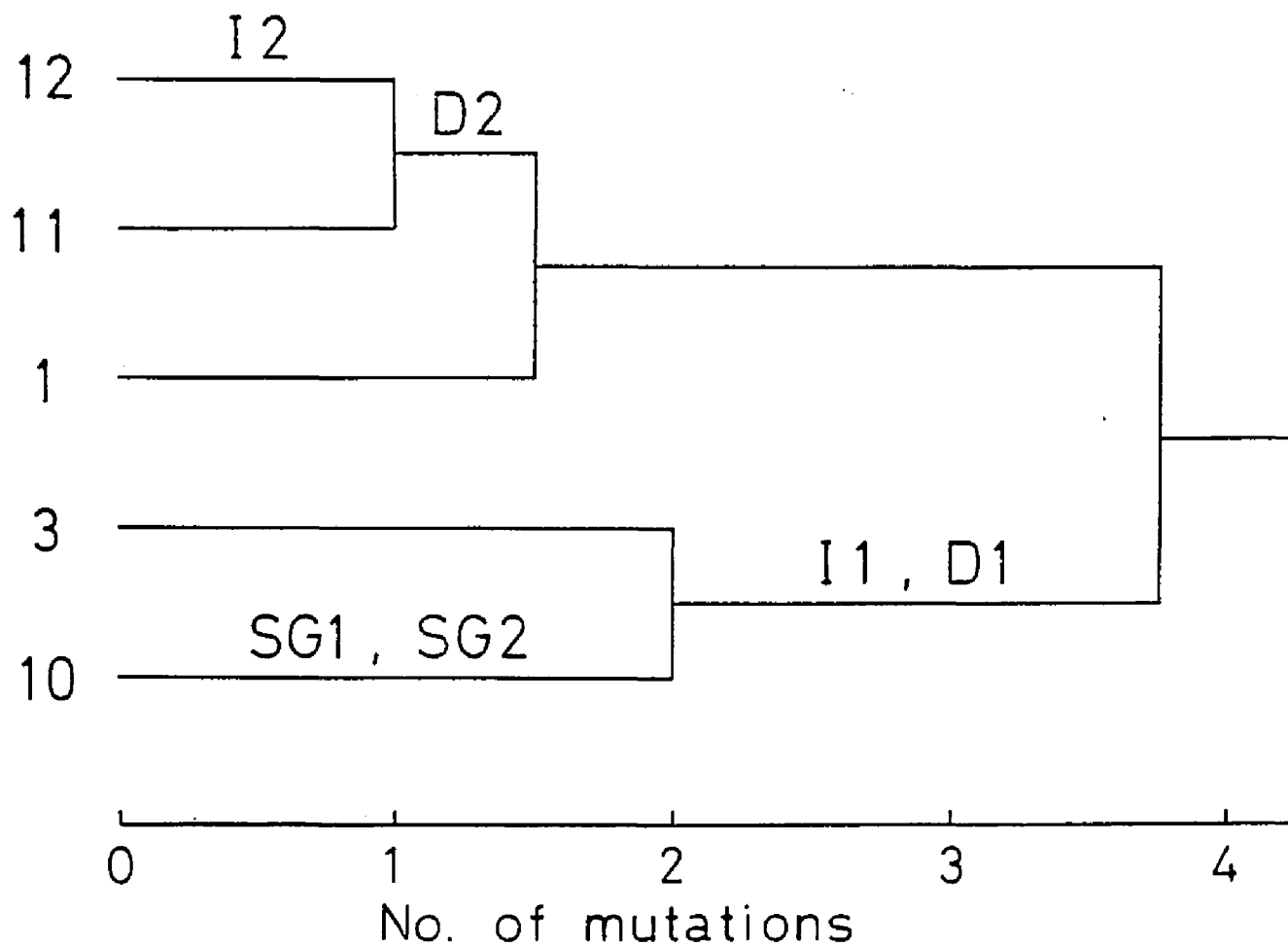


Fig. 18. Dendrogram showing the phylogenetic differentiation of five chloroplast genome types found in 68 cultivars of *O. sativa*. Mutations occurred in each branch are indicated. The symbols for mutations are given in Fig. 17.

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:

$$\text{Percentage of common fragments} = \frac{2b}{(a + a')} \times 100,$$

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.

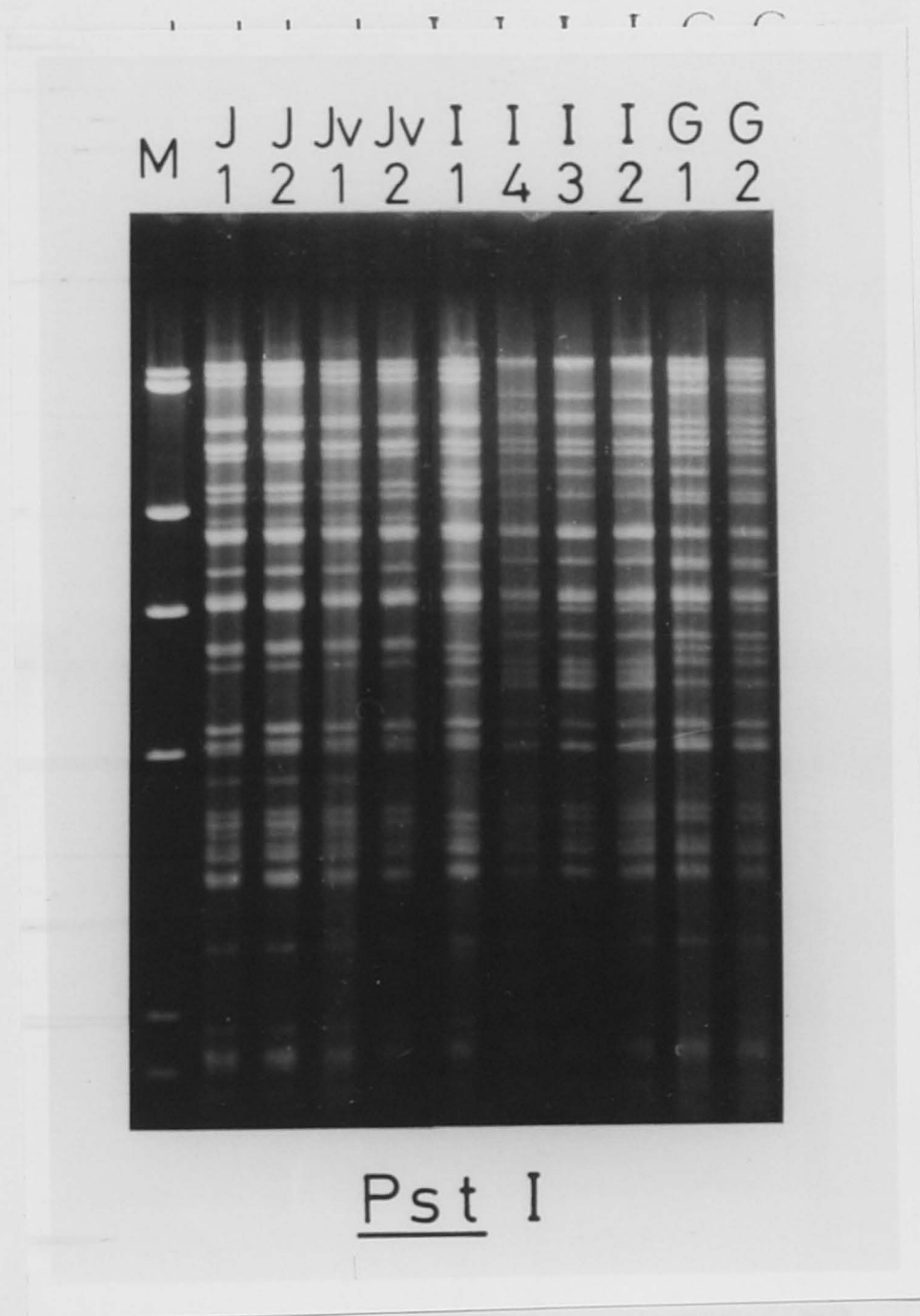
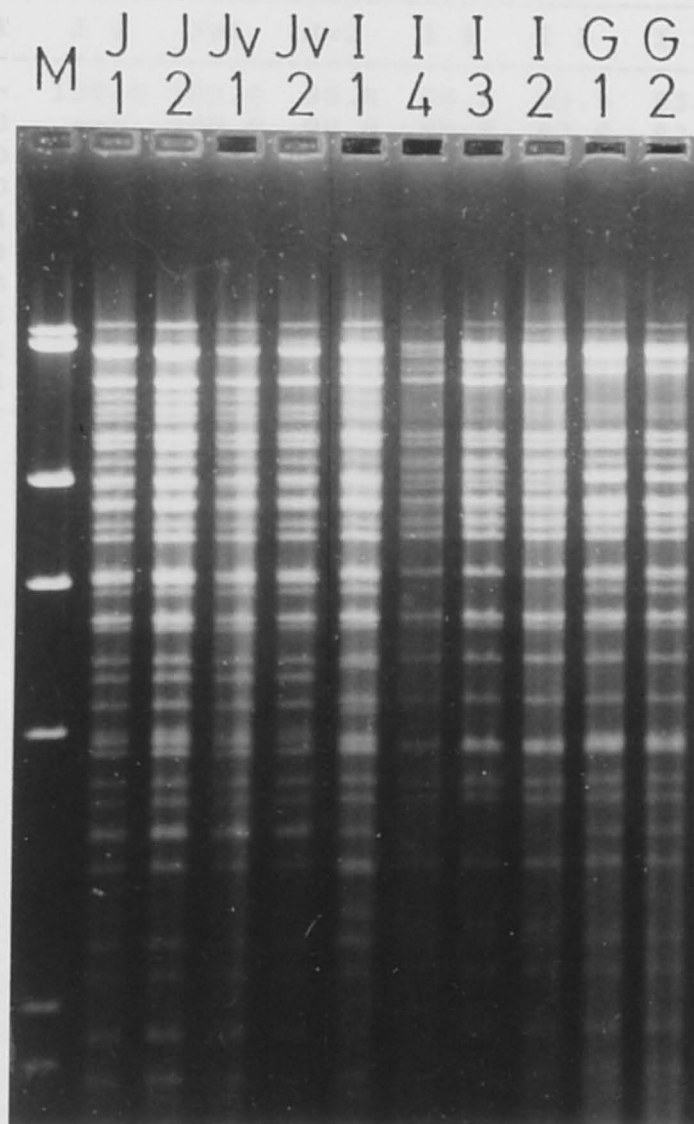


Fig. 19. PstI restriction fragment patterns of mtDNA from ten cultivars of Oryza.
M: Lambda DNA digested with HindIII.

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

Code	J 1	J 2	Jv 1	Jv 2	I 1	I 2	I 3	I 4	G 1	G 2
J 1.	---									
J 2.	136	---								
Jv1.	330	83.5	---							
Jv2.	330	83.5	83.5	---						
I 1.	334	83.5	83.5	83.5	---					
I 2.	333	86.1	86.4	86.4	86.1	---				
I 3.	333	100.0	86.9	86.9	100.0	86.9	---			
I 4.	333	100.0	86.9	86.9	100.0	86.9	86.9	---		
G 1.	343	344	352	352	344	352	352	100.0	---	
G 2.	343	344	352	352	344	352	352	100.0	100.0	---



Pvu II

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	---

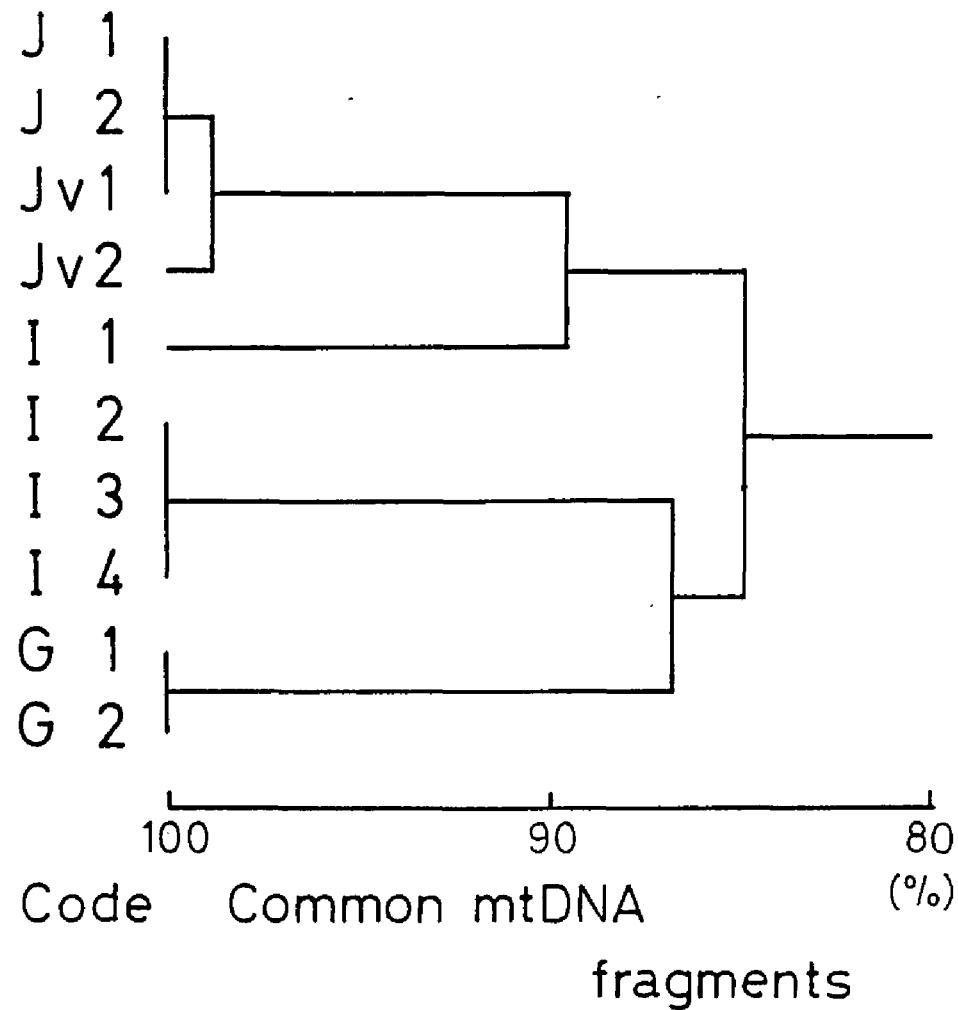


Fig. 21. Dendrogram showing genetic relationships between mitochondrial genomes of ten cultivars of *Oryza* based on the percentage of common mtDNA fragments observed in the restriction fragment patterns of their mtDNAs.

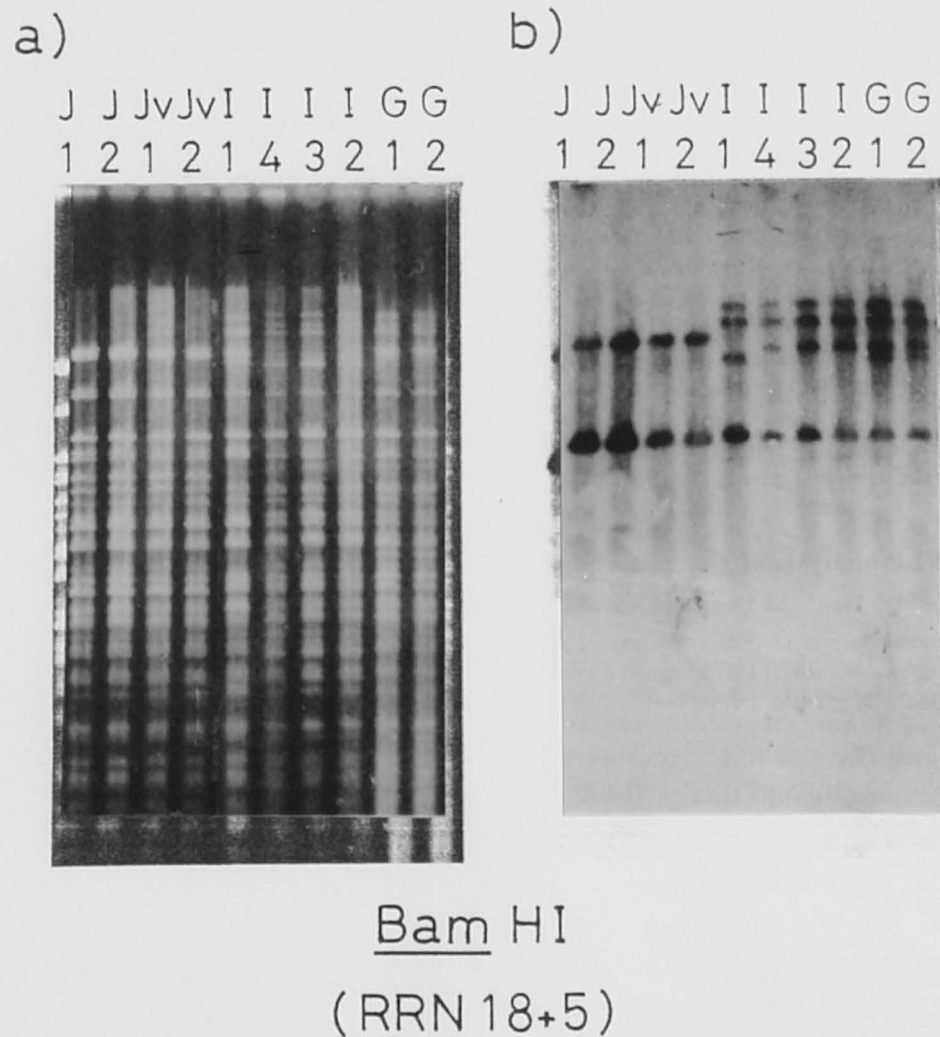


Fig. 22. a) BamHI restriction fragment patterns of mtDNA from ten cultivars of Oryza. b) Southern hybridization of the same patterns with wheat RRN18&5 probe.

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	---

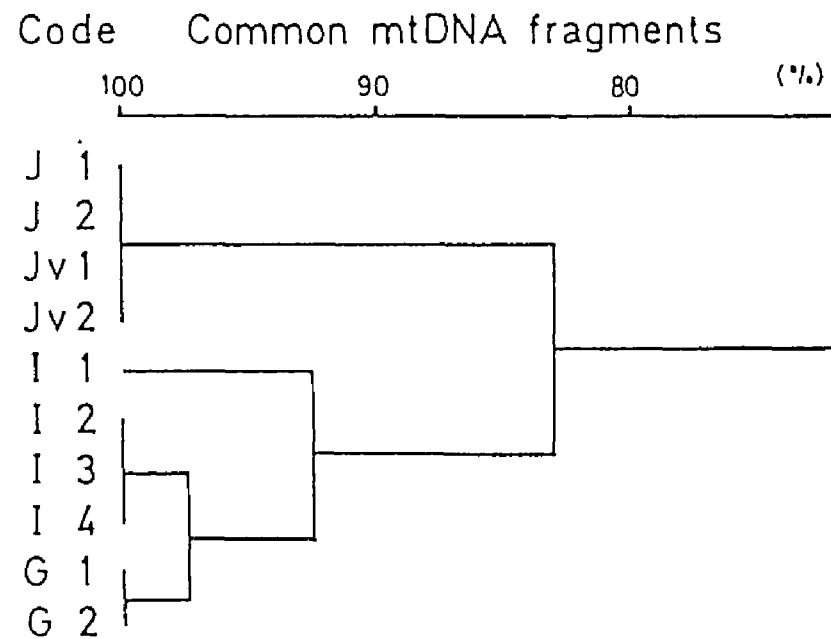


Fig. 23. Dendrogram showing genetic relationships between mitochondrial genomes of ten cultivars of *Oryza* based on the percentage of common fragments observed in their Southern hybridization patterns of their mtDNAs.

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:

$$D = -\ln [J_{XY} / (\underline{J}_X \underline{J}_Y)^{1/2}],$$

where $\underline{J}_X$, $\underline{J}_Y$ and $\underline{J}_{XY}$ are the arithmetic means, over all loci, 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 i th locus in populations X and Y , respectively). In the present analysis, $\underline{J}_X$ and $\underline{J}_Y$ correspond to the numbers of total fragments in accessions X and Y , respectively, and $\underline{J}_{XY}$ is the number of common fragments observed between accessions X and Y .

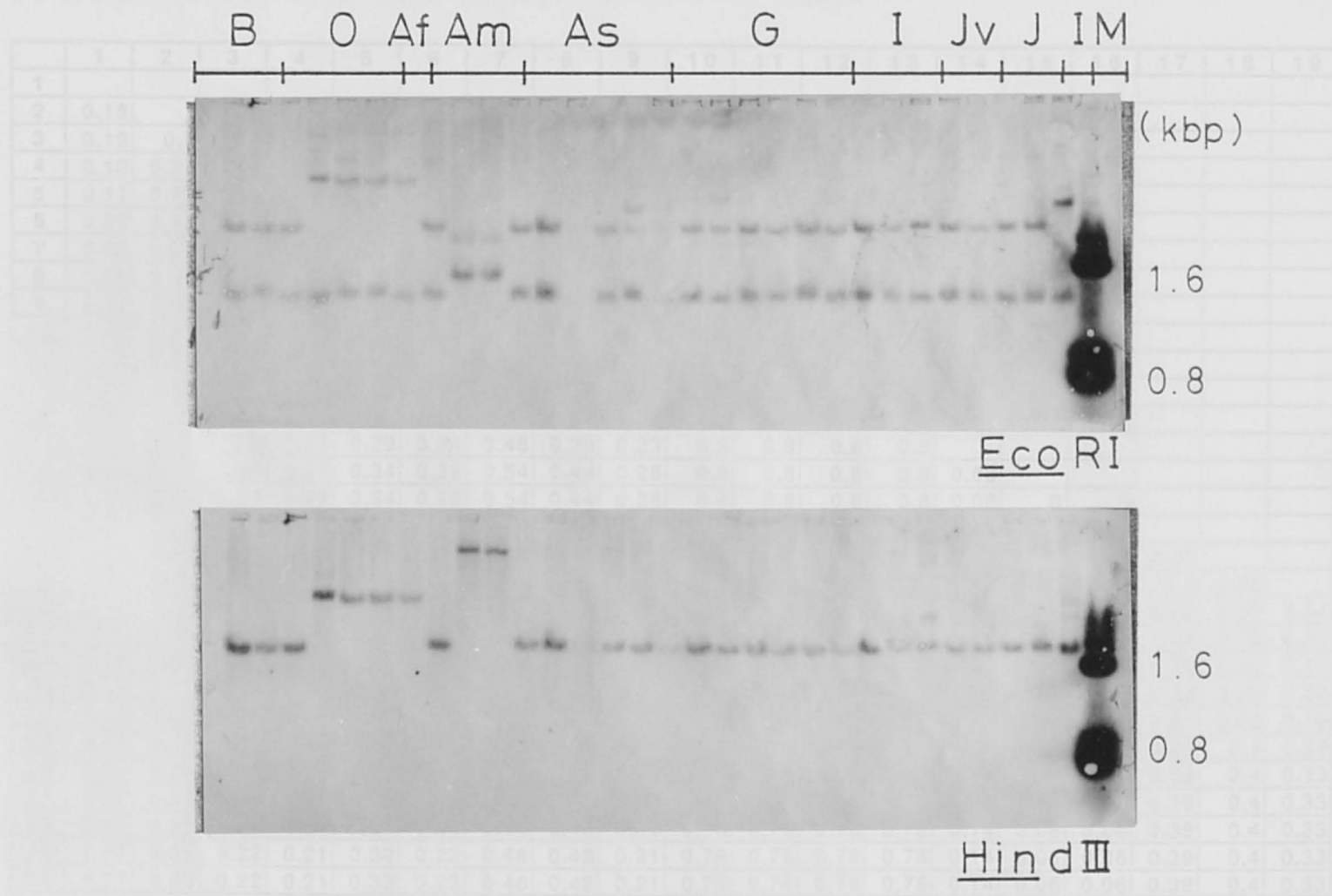
The number of total fragments ranged from 22 to 31 among 30 accessions. Table 21 gives the genetic distances between 30 accessions, based on which a dendrogram was constructed using a complete linkage clustering method (Sneath and Sokal 1973) to show the differentiation of the nuclear genome among 30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



Note) Accession numbers (see RG 144 indicated in the top row and the left end)

Fig. 24. Southern hybridization patterns of the *Eco*RI- and *Hind*III-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. breviliquata*. 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	0.11																	
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	

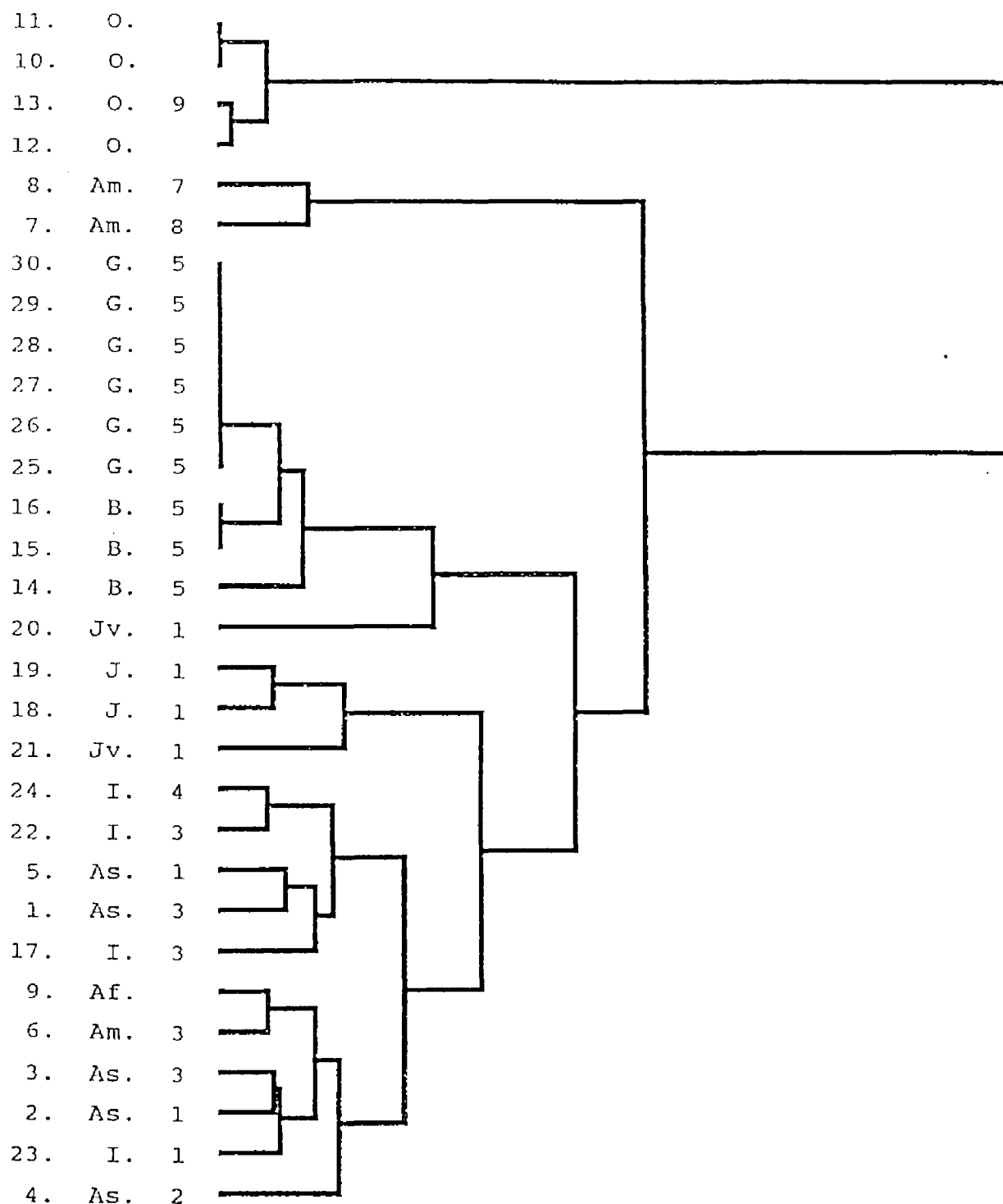


Fig. 25. Dendrogram showing genetic relationships of nuclear genome between 30 accessions of A-genome diploid species of *Oryza*. The first column on the left gives accession number (see Table 4), the second column abbreviated taxon (see Fig. 24) and the third column chloroplast genome type of these 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 *Hind*III and *Pst*I. 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 *Eco*RI and *Hind*III. 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⁾	
					<u>HindIII</u>	<u>PstI</u>
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

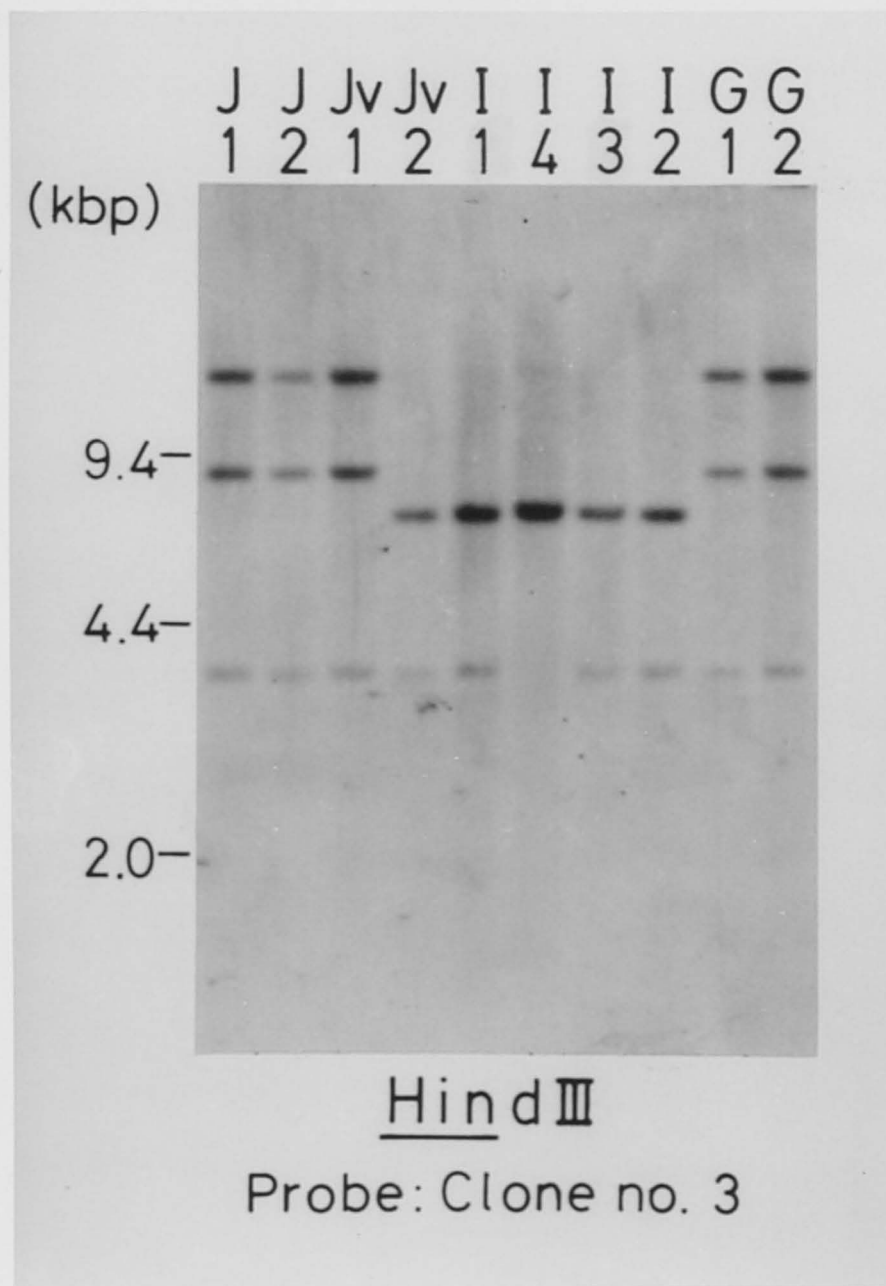
1) N: Nipponbare, I: IR36.

2) P: PstI site, H: HindIII site.

3) M: Monomorphic, P: Polymorphic.

--: Not tested.

Table 23. Number of the total fragments (below diagonal) and percentage of common fragments (above diagonal) observed between



	G 1	G 2
4	88.3	86.7
4	88.3	86.7
4	88.3	86.7
7	84.0	84.0
7	82.4	82.4
9	79.0	79.0
11	60.9	67.2
11	79.3	79.3
11	98.3	
11	118	

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	---

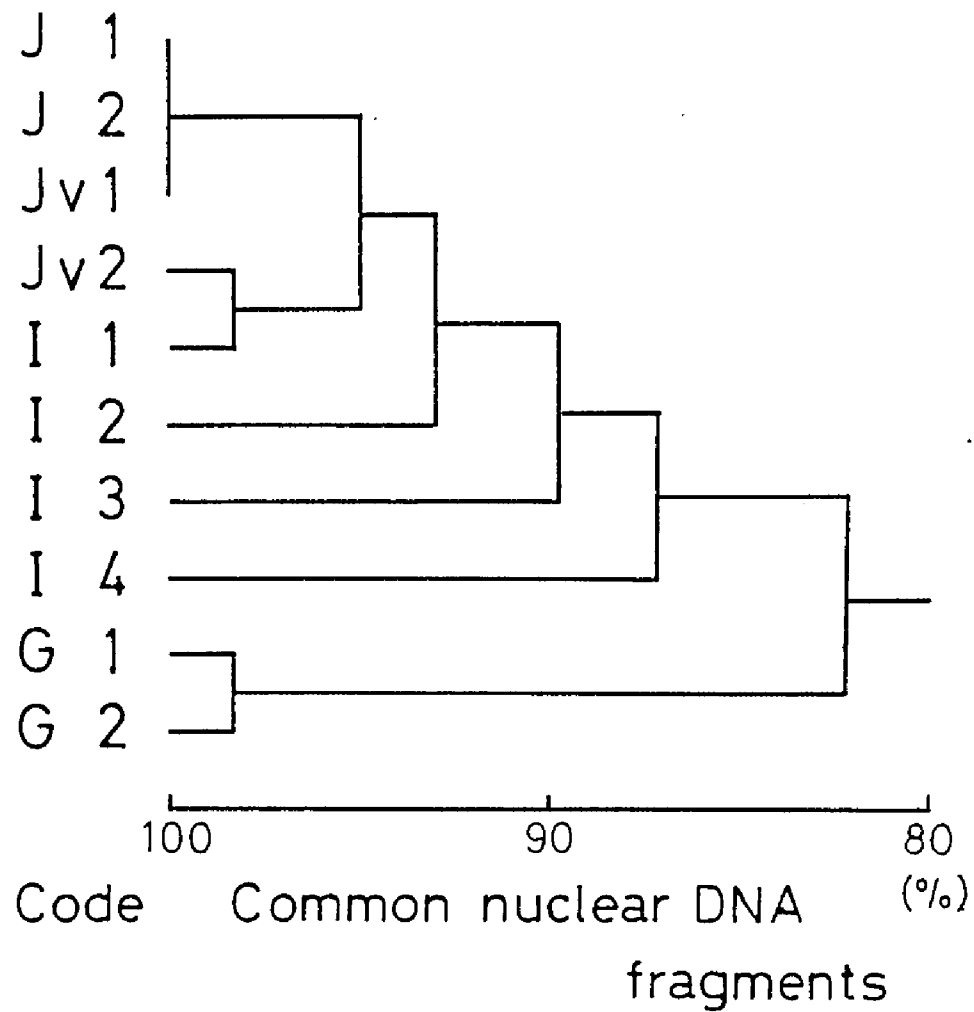


Fig. 27. Dendrogram showing genetic relationships between nuclear genomes of ten cultivars of *Oryza* based on the percentage of common nuclear DNA fragments.

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

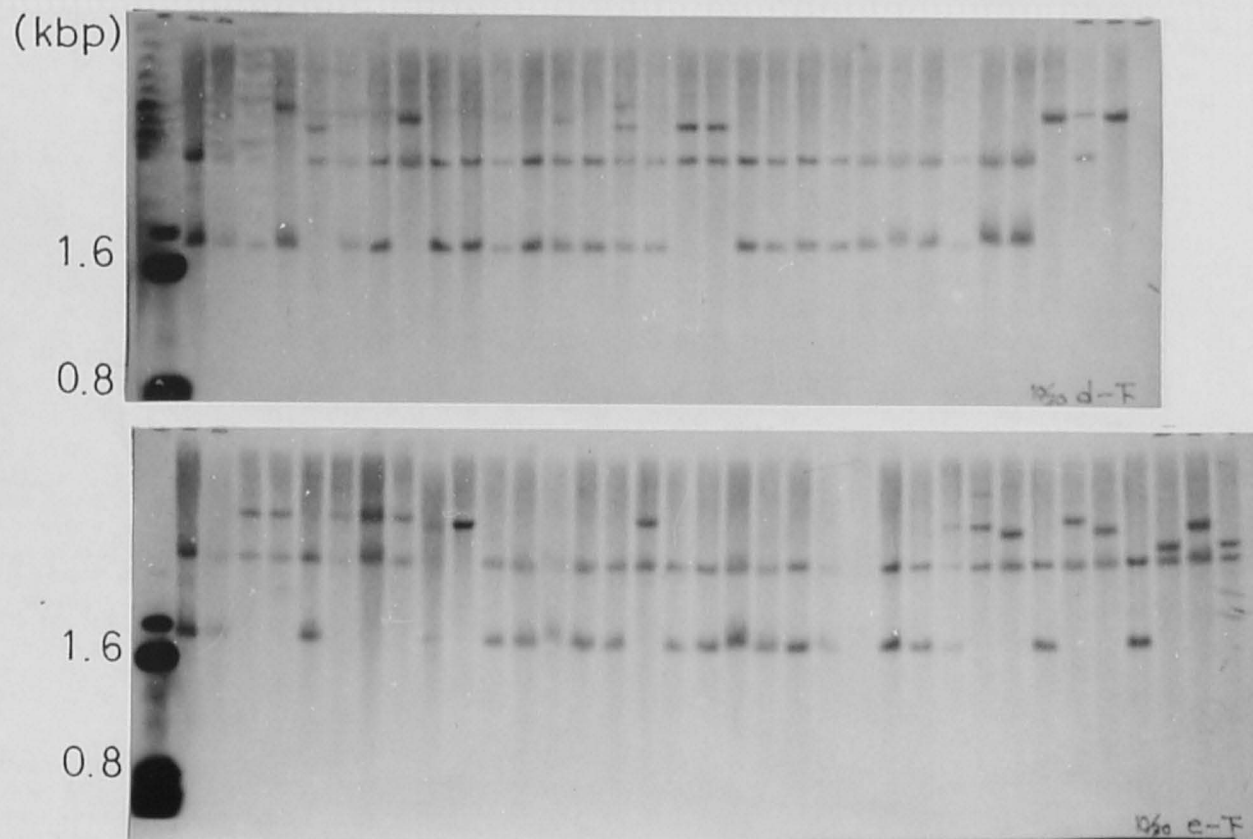
No.	Acc.no.	Glaszmann's data ¹⁾					Present results				
		A	B	C	D	E	A	B	C	D	E
24	7722	1	1	2	2	2	1	2	2	2	2
30	12881	2	4	0	1	2	2	4	0	1	1
39	27590	2	1	6	1	2	2	1	6	1	2
40	27762	1	1	5	2	1	2	2	5	2	1
41	27798	2	1	0	1	1	2	1	0	1	2
56	43394	2	1	1	1	1	2	1	1	0	1
73	58930	2	4	4	1	2	1	1	1	2	1
97	26872	2	1	1	1	1	2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

Eco RI



RG 214

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

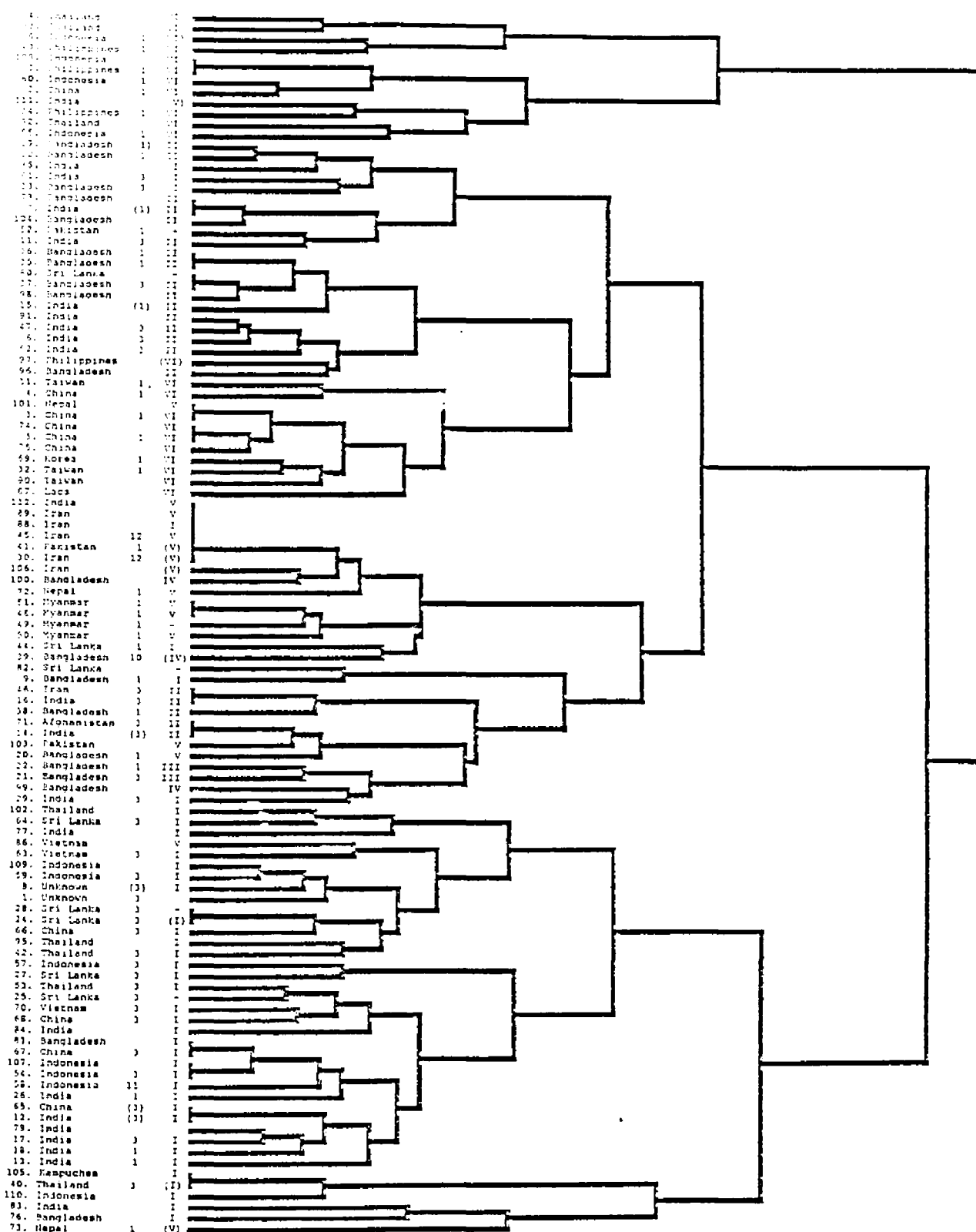


Fig. 29. Dendrogram showing genetic relationships of nuclear genome between 112 cultivars *O. sativa* as revealed by RFLP analysis of their nuclear DNAs. The first column gives their accession number (see Table 3), the second column origin, the third column chloroplast genome type and the forth column 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 Q. perennis contained seven chloroplast genome types, whereas Q. 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 Q. perennis appears to be extensive, and seems to be related to its wide geographical distribution.

The African and Oceanian forms of Q. 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 Q. perennis, are quite different from those of Q. sativa, Q. glaberrima and other accessions of Q. perennis. Their and the present results indicate that the American form of Q. 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 genome 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

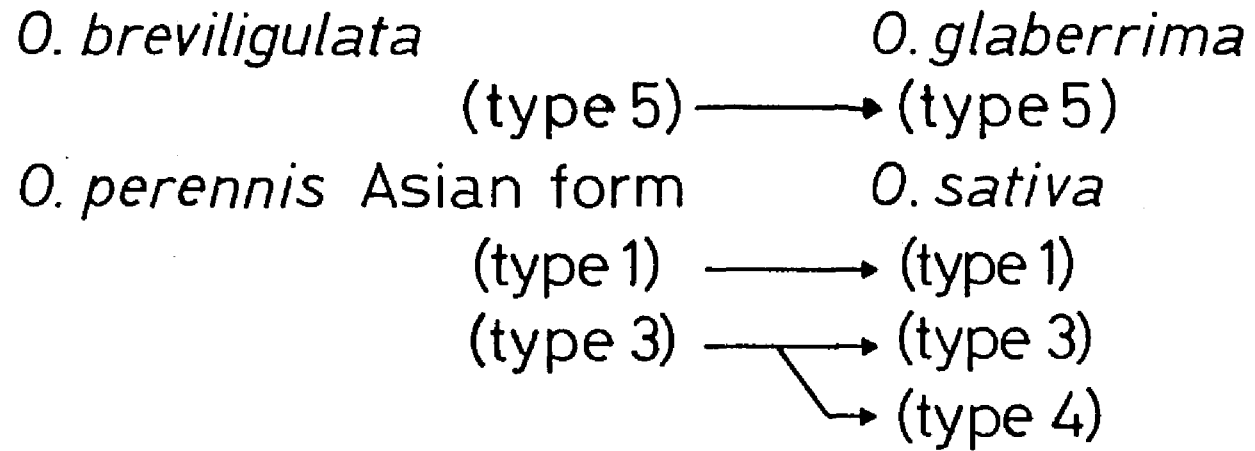


Fig. 30. Possible origins of two cultivated species.

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) selected 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	<u>Bam</u> HI	<u>Bgl</u> II	<u>Eco</u> RI	<u>Eco</u> RV	<u>Pvu</u> II	<u>Sal</u> I	<u>Sma</u> I	<u>Xho</u> I	<u>Hin</u> dIII	<u>Pst</u> I	<u>Xba</u> 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.

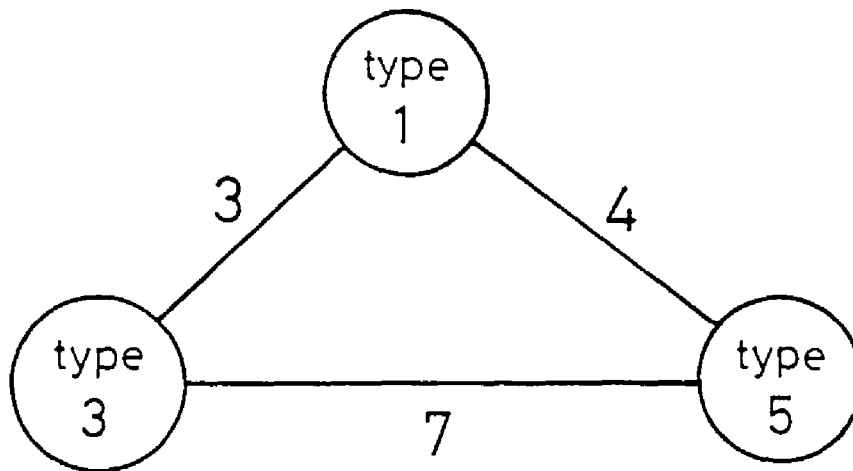
So far as the present results are concerned, restriction endonuclease analysis proved to be more effective to detect ctDNA variation than nucleotide sequence analysis, because seven mutational events were discovered in 1194 bp by the former method, whereas no mutation was found in 1738 bp which were 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)

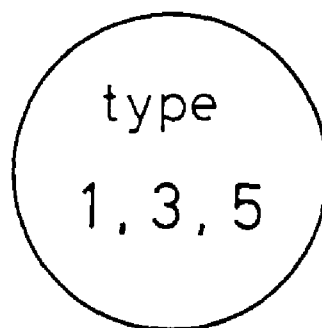


Fig. 31. Comparison of the results between restriction endonuclease analysis and sequence analysis with type 1, 3 and 5 chloroplast genomes.

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 hybridization 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 genome 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 mitochondrial 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 Q. perennis Oceanian form (Table 7). These results indicate Q. 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 Q. perennis American form also differentiated greatly from others. Their chloroplast genome types (types 7 and 8) were unique for this form (Table 7). Therefore, Q. perennis American form might have also achieved unique differentiation from other forms and species.

Single accession of both Q. perennis American form and African form formed a cluster with Q. perennis Asian form and Q. sativa ecosp. Indica, and are considered as exceptional types. The accession of Q. perennis American form had type 3 chloroplast genome, which was mainly found in Q. perennis Asian form and Q. 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 Q. perennis African form, its ctDNA data are not available. However, most of Q. 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: Q. perennis Asian form formed a large cluster with Q. sativa ecosp. Indica, Q. sativa ecosp. Japonica and Javanica composed satellite groups. Q. breviliquata and Q. glaberrima were included in the same cluster.

Magnitude of nuclear genome differentiation in *Q. 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

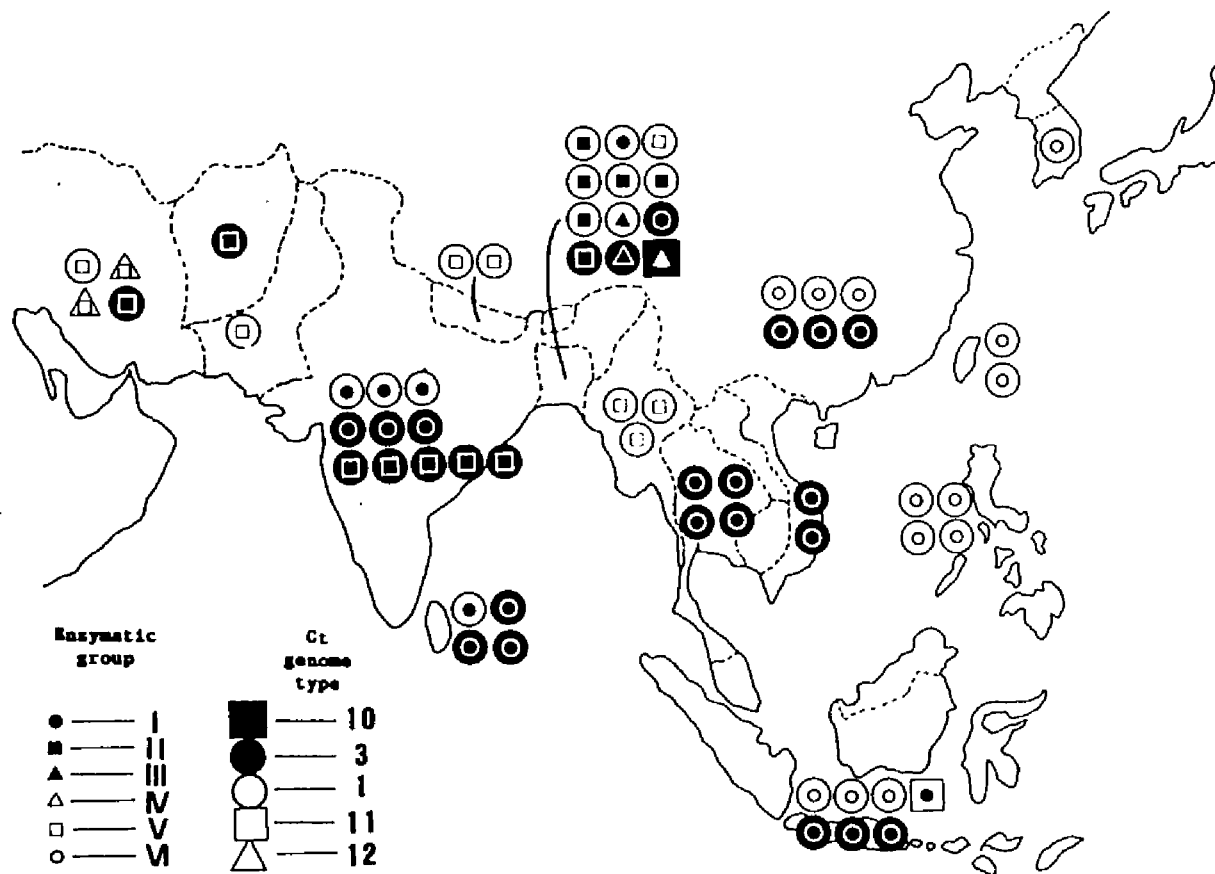


Fig. 32. Geographical distribution of 64 cultivars of *O. sativa* having different enzymatic (I - VI) and chloroplast genome types (1, 3, 10, 11 and 12).

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 type 1 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.

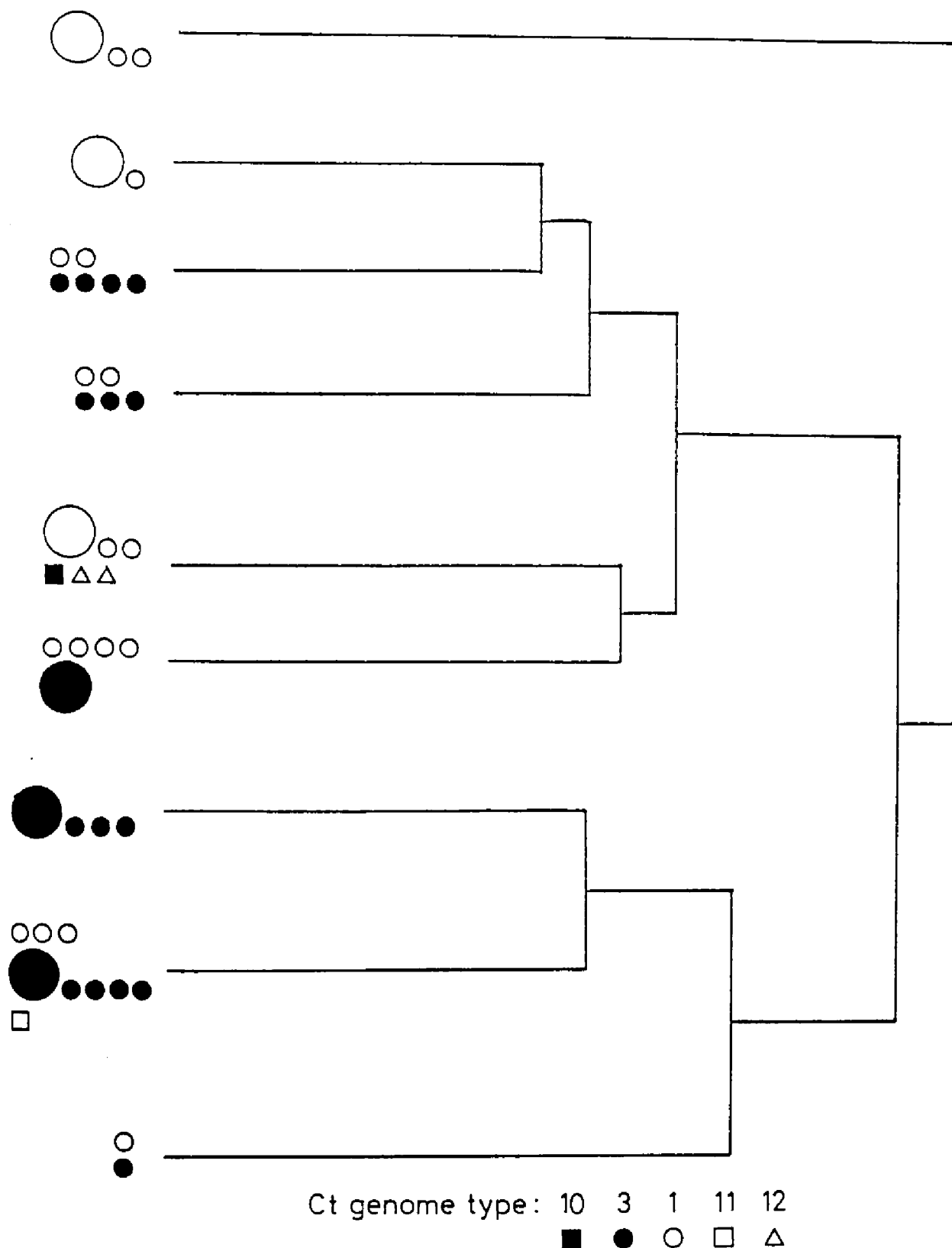


Fig. 33. A Dendrogram showing genetic relationships between nine major clusters of nuclear DNAs with the number of cultivars and their chloroplast genome types belonging to each cluster in *Q. sativa*. Big and small symbols represent five and one cultivars, respectively.

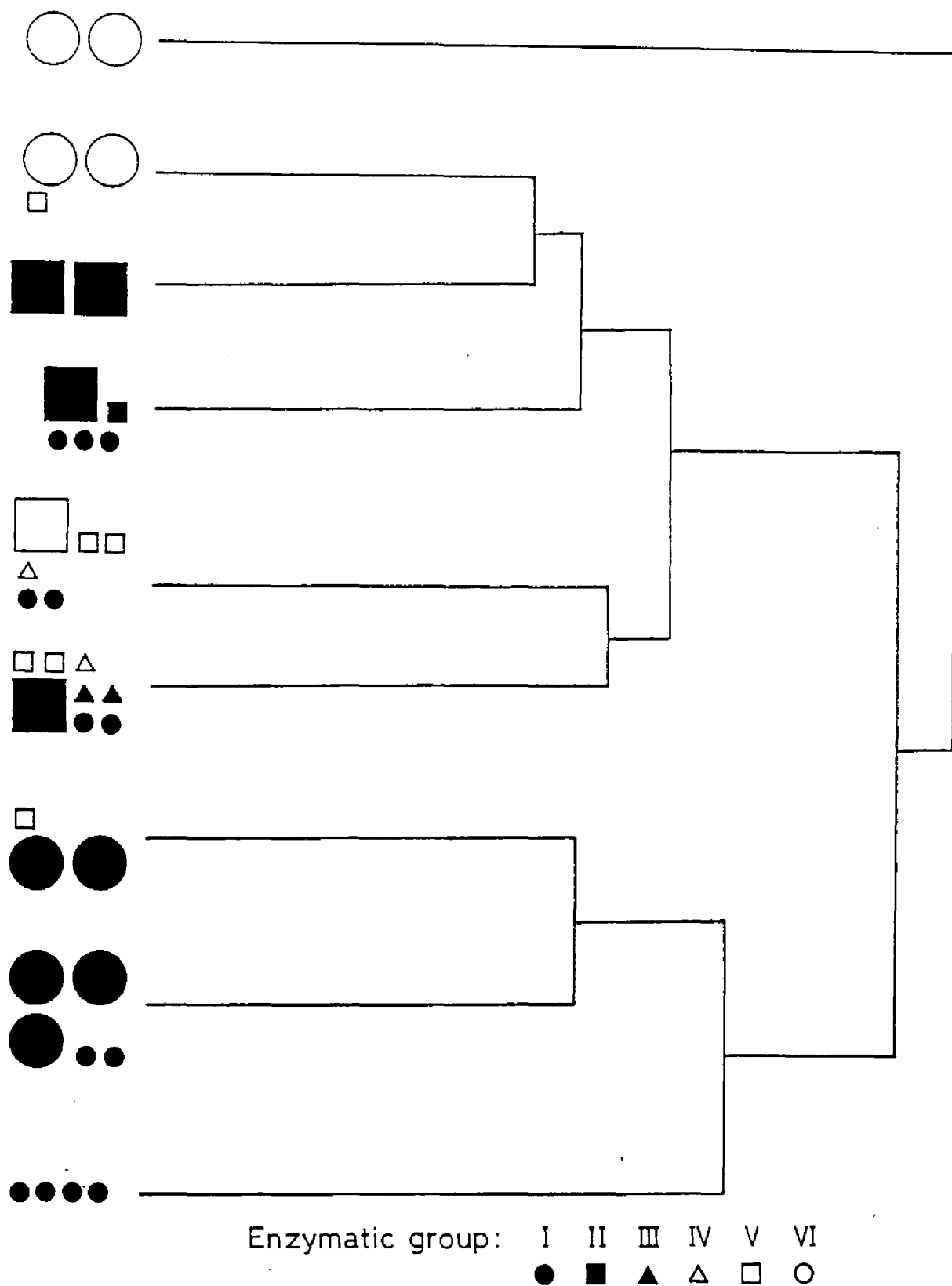


Fig. 34. Dendrogram showing genetic relationships between nine major clusters of nuclear DNAs in *O. sativa*, with the number of cultivars and their enzymatic groups (Glaszmann 1985), belonging to each cluster. Big and small symbols represent five and one cultivars, respectively.

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.

Restriction 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 American 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, Sall, 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.

ACKNOWLEDGEMENTS

I wish to my deepest appreciation to Professor K. Tsunewaki, Laboratory of Genetics, Faculty of Agriculture, Kyoto University, for his continuous guidance throughout this work and for reviewing the manuscript of this thesis.

I am greatly indebted to Dr. G. S. Khush, Plant Breeding, Genetics and Biochemistry Division (PBGB), International Rice Research Institute (IRRI), Philippines, for his kind support and for accepting me to conduct my thesis research in IRRI.

Thanks are also due to Dr. A. Hirai, Faculty of Agriculture, Nagoya University, Dr. T. Terachi, Faculty of Engineering, Kyoto Sangyo University, Dr. N. Mori, Faculty of Agriculture, Kobe University, Mr. T. M. Ikeda, Faculty of Agriculture, Kyoto University, and Dr. R. Terauchi, International Institute of Tropical Agriculture, Nigeria, for their guidance in the experiment.

I am grateful to Dr. D. S. Brar, Dr. S. McCouch and Dr. G. Second, PBGB, IRRI, and Dr. R. Nelson, Plant Pathology Division, IRRI, for their kind advice and support and for affording facilities in IRRI.

My deepest gratitude is due to Dr. J. C. Glaszmann, Institut de Recherches Agronomiques Tropicales et de Cultures Vivrieres, France, for kindly providing useful information.

I wish to express my appreciation to Dr. T. T. Chang, International Rice Germplasm Center, IRRI, Dr. Y. Sano, National Institute of Genetics, Dr. T. Yabuno, College of Agriculture, University of Osaka Prefecture, Dr. S. Tsuji, Smitomo Chemicals Co. Ltd., and Dr. T. Horie and Dr. Y. Peng, Faculty of Agriculture, Kyoto University, for supplying the materials used in the study.

Probe DNAs were kindly provided by Dr. A. Hirai and Dr. K. Nakamura, Faculty of Agriculture, Nagoya University, Dr. F. Quetier, University of Paris, France, Dr. S. D. Tanksley, Cornell University, USA, and Dr. S. McCouch, PBGB, IRRI: to whom my sincere thanks are expressed.

Cordial thanks are due to Mr. O. Panaud, PBGB, IRRI, who shared hard time with me in

early molecular biology work in IRRI, and to Mr. B. G. de los Reyes, who kindly taught me techniques of Isozyme analysis.

Special appreciation is to Japanese staffs and an English staff in IRRI, Dr. I. Watanabe, Dr. R. Ikeda, Dr. H. Koganezawa, Dr. M. Yamauchi and Dr. D. A. Vaughen, for their encouragement and for occasionally supplying Japanese dishes in the Philippines.

Special thanks are due to Japanese scholars in IRRI, Dr. N. Endo (Biotech Research Center, Taisei Corporation), Mr. N. Kobayashi, and Mr. H. Yasui (Faculty of Agriculture, Kyushu University), for their friendship and for organizing small Japanese scholar society in IRRI.

I also thank all the members in Laboratory of Genetics, Faculty of Agriculture, Kyoto University, and in RFLP, Isozyme and Cytogenetics Laboratories, PBGB, IRRI, for their continuous encouragement and friendship during the study.

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