Genetic Resources and Crop Evolution **39**: 125–140, 1992.

Mitochondrial DNA RFLP in genus *Oryza* and cultivated rice

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Received 8 April 1992; accepted 17 September 1992

Key words: genetic resources, mitochondrial inheritance, mitochondrial DNA RFLP, Oryza, phylogeny, rice

Summary

Ninety-three accessions representing 23 species from the genus *Oryza* were surveyed for restriction fragment length polymorphism (RFLP) in mitochondrial (mt) DNA by probing total DNA with 15 known mt sequences cloned in plasmids from higher plants, and five mt genomic cosmid clones from maize. Very low levels of intra-specific and even intra-cytologically-defined nuclear genome mt DNA RFLP were found. High between-genome differentiation appeared, suggesting phylogenetic relationships consistent with data from previous nuclear and chloroplast (cp) DNA studies. Parallel inheritance of cp and mt DNA was found. There was one major exception: the mt DNA of the allotetraploid CD genome is apparently equally related to two putative diploid progenitors, which is suggestive of an interspecific recombination.

RFLP in mt DNA was also probed in 82 cultivars, with four plasmid probes. Some bands not seen in the wild species appeared in *O. sativa*, with intra-specific polymorphism relatively higher than in the wild species. The pattern of variation paralleled that at the cp DNA level between the *indica* and *japonica* subspecies.

Introduction

Molecular evaluation of genetic diversity is a way to study the amount and partitioning of genetic variability in cultivated species and their wild relatives, as well as gene flow across their reproductive barriers. It also clarifies their phylogenetic relationships and can provide a rationale for choosing strategies for breeding and conservation of genetic resources.

Genetic information is encoded in three cellular compartments with partly autonomous reproduction as well as contrasting evolutionary characteristics: nucleus (n), chloroplast (cp), and mitochondria (mt). A study of these three DNAs should give complementary evaluation of genetic diversity and phylogenetic relationships. We report here a study of mt DNA RFLP in the whole genus *Oryza*, including cultivated rice.

In rice, the mt genome was shown to differ between *indica* and *japonica* subspecies of cultivated rice (Chowdhury et al., 1988), between normal and male sterility inducing cytoplasm (Mignouna et al., 1987; Kadowaki et al., 1988a), and to have accumulated variation in restriction patterns in long-term tissue-cultured lines (Chowdhury et al., 1988, 1990). A recent estimate of rice mt genome size is around 528 kb (Hirai et al., 1991). Isozyme diversity, single copy n DNA restriction fragment length polymorphism (RFLP),

and cp DNA RFLP have already been used to address molecular evaluation of genetic diversity and phylogeny, particularly within the section *Oryza*, genus *Oryza* (Second 1984, 1985; Dally & Second, 1990; Wang et al., 1991). The present paper aims to give a comparable picture of diversity at the mt DNA level.

Based on molecular marker studies confirming or clarifying earlier morphological or cytological investigations, a natural classification of genus *Oryza* can now be proposed. A most diverse section *Oryza* comprises two natural groups of species for which genomes have been characterized at the level of chromosome pairing in F1 hybrids (see Vaughan, 1989, 1990 and Table 1 for species names). The group Sativa comprises cultivated rice and its closest wild relatives distributed throughout the tropics, all diploids with a single genome A. The group Latifolia (or Officinalis) comprises species also distributed throughout the tropics but with diploid and allotetraploid genomes: B in Africa, C and BC in Asia and Africa, E in Australia, and CD in America (Second, 1985; Vaughan, 1989; Dally & Second, 1990; Wang et al.,

Table 1. 93 accessions studied in genus *Oryza*. Accession codes and the revised classification are given as in Wang et al. 1992. Cytological genomes (Capitals) and species symbols (lower-case) are given in column G; the total number of bands observed with plasmid probes is given in column Bd

Acc.	Acc. Code	Classification	G	Origin	Bd
1	51064	*O sativa.* L.ssp. *indica* Kato	Asa	Sri Lanka	20
2	17054	*O. sativa.* L.ssp. *japonica* Kato	Asa	Taiwan	19
3	DS14	*O. rufipogon* Griff.	Aru	West India	20
4	W162	*O. rufipogon*	Aru	Thailand	20
5	W1699	*O. rufipogon*	Aru	Thailand	20
6	W133	*O. rufipogon*	Aru	India	20
7	PI 88-791	*O. rufipogon*	Aru	Thailand	20
8	W1669	*O. rufipogon*	Aru	India	20
9	W1654	*O. rufipogon*	Aru	China	20
10	W1655	*O. rufipogon*	Aru	China	20
11	W135	*O. rufipogon*	Aru	India	20
12	W593	*O. rufipogon*	Aru	Malaysia	21
13	103822	*O. rufipogon*	Aru	China	20
14	103831	*O. rufipogon*	Aru	Bangladesh	20
15	DR38	*O. rufipogon*	Aru	West India	18
16	W555	*O. rufipogon*	Aru	Sri Lanka	20
17	101508	*O. rufipogon*	Aru	India	20
18	100968	*O. glumaepatula* Steud	Aglu	Surinam	20
19	W1185	*O. glumaepatula*	Aglu	Surinam	20
20	OR7	*O. meridionalis* Ng	Ame	Australia	20
21	OR10	*O. meridonalis*(a)	Ame	Australia	20
22	W1627	*O. meridionalis*	Ame	Australia	20
23	101147	*O. meridionalis*	Ame	Australia	20
24	OR39	*O. meridionalis*(a)	Ame	Australia	20
25	OR54	*O. meridionalis*	Ame	Australia	20
26	102201	*O. glaberrma* Steud	Agl	West Africa	20
27	100122	*O. barthii* A. Chev.	Aba	Gambia	20
28	WB01	*O. barthii*	Aba	Botswana	20
29	TL81	*O. longistaminata* A. Chev. et Roehr.	Alon	Mali	21
30	101378	*O. longistaminata*	Alon	Mali	21
31	1LL 116	*O. longistaminata*	Alon	Mali	21
32	IL 52	*O. longistaminata*	Alon	Ivory Coast	21
33	WL02	*O. longistaminata*	Alon	Botswana	21
34	UL 12-6	*O. longistaminata*	Alon	Cameron	21
35	YL 244	*O. longistaminata*	Alon	Guinea	21
36	ZL 14	*O. longistaminata*	Alon	Zambia	21

Table 1. Continued.

37	CL7-2	*O. longistaminata*	Alon	Senegal	21
38	W1590	*O. punctata* Kotschy *ex* Steud (*2n*)	Bpu	Cameroun	20
39	W1515	*O. punctata* (*2n*)	Bpu	Tanzania	20
40	TP43	*O. punctata* (*2n*)	Bpu	Tchad	20
41	101089	*O. minuta* J. S. Presl. *ex* C. B. Presl.	BCmi	Phillippines	20
42	101125	*O. minuta*	BCmi	Phillippines	20
43	101141	*O. minuta*	BCmi	Phillippines	20
44	103865	*O. minuta*	BCmi	Phillippines	20
46	100181	*O. minuta*	BCpu	Phillippines	20
45	W1331	*O. punctata* Kotschy *ex* Steud. (*4n*)	BCpu	Phillippines	21
47	IP 27	*O. punctata* (*4n*)	BCpu	Ivory Coast	21
48	W1408	*O. punctata* (*4n*)	BCpu	Nigeria	21
49	101409	*O. punctata* (*4n*)	BCpu	Ghana	21
50	100180	*O. punctata* (*4n*)	BCpu	Africa	21
51	100957	*O. malampuzhaensis* Krish. *et* Chand	BCma	India	20
52	103410	*O. rhizomatis* D.A. Vaugh.	Crh	Sri Lanka	20
53	103421	*O. rhizomatis*	Crh	Sri Lanka	20
54	101422	*O. eichingeri* A. Peter	Cei	Uganda	22
55	W1526	*O. eichingeri*	Cei	Uganda	21
56	IP7	*O. eichingeri*	Cei	Ivory Coast	21
57	101425	*O. officinalis* Wall. *ex* Watt	Cof	Uganda	22
58	100896	*O. officinalis*	Cof	Thailand	22
59	101150	*O. officinalis*	Cof	E. Malaysia	22
60	DO4	*O. officinalis*	Cof	India	22
61	W1278	*O. officinalis*	Cof	Sarawak	22
62	104618	*O. officinalis*	Cof	China	22
63	105392	*O. officinalis*	Cof	China	22
64	105393	*O. officinalis*	Cof	China	22
65	105394	*O. officinalis*	Cof	China	22
66	105395	*O. officinalis*	Cof	China	22
67	105396	*O. officinalis*	Cof	China	22
68	101395	*O. alta* Swall.	CDal	S. America	23
69	101405	*O. grandiglumis* (Doell) Prod.	CDgr	Brazil	23
70	ch 83-3	*O. latifolia* Desv.	CDla	?	22
71	W1168	*O. latifolia*	CDla	Cuba	23
72	W1144	*O. latifolia*	CDla	S. America	23
73	100914	*O. latifolia*	CDla	Mexico	23
74	100963	*O. latifolia*	CDla	Guatemala	23
75	OA4	*O. australiensis* Domin	Eau	Australia	21
76	OA27	*O. australiensis*	Eau	Australia	21
77	OA36	*O. australiensis*	Eau	Australia	21
78	100882	*O. australiensis*	Eau	Australia	21
79	EY25	*O. brachyantha* A. Chev. *et* Roehr.	Fbra	Tanzania	18
80	W654	*O. brachyantha*	Fbra	West Africa	18
81	W656	*O. brachyantha*	Fbra	West Africa	18
82	W615	*O. granulata* Nees *et* Arn. *ex* Watt	2n	Burma	34
83	W3	*O. granulata*	2n	India	34
84	W5	*O. granulata*	2n	Sri Lanka	34
85	W67	*O. granulata*	2n	Thailand	34
86	W609	*O. meyeriana* Baill.	2n	China	34
87	W1348	*O. granulata*	2n	Borneo	34
88	W1228	*O. longiglumis* Jansen	4n	New Guinea	21
89	W1220	*O. longiglumis*	4n	New Guinea	21
90	100821	*O. ridleyi* Hook. f.	4n	Thailand	21
91	W2033	*O. ridleyi*	4n	Thailand	21
92	W604	*O. ridleyi*	4n	Malaysia	21
93	W1	*O. ridleyi*	4n	Thailand	21

(a): perennial life form.

1991). The genome D has not been identified at the diploid level. Its origin is still controversial. While conventional interpretation of RFLP marker data points to an ancient origin in America (Wang et al., 1991), Second (1991) has alternatively proposed an origin through hybridization between Old World species and their rapid diversification since human colonization of tropical America. The rest of genus *Oryza* is composed of the two less diverse *O. meyeriana* and *O. ridleyi* complex and one isolated species, *O. brachyantha.*

Materials and methods

Plant materials, isolation of DNA, Southern blotting and hybridization techniques. We reused Southern blotted membranes previously used for single-copy n DNA RFLP survey (Wang et al., 1991 and unpublished). Unless otherwise stated, they were obtained from total DNA restricted with *Eco*RI. The plant materials are thus the same (see Tables 1 and 5 for accession numbers and classification) and the same hybridization technique was used. To test for cross hybridization with cp DNA, we used Southern blotted membranes that had been prepared from purified cp DNA restricted with various enzymes (Dally & Second, 1990). When variable bands were seen, they were compared with bands observed on the same filters from hybridization with cp DNA clones (Shimada et al., 1989) altogether representing the total cp DNA molecule (unpublished data), to score possible homology. Autoradiographs were exposed for three days or less.

Probes. Two sets of mt DNA probes were used as outlined in Table 2. One was composed of 15 known DNA sequences inserted in a plasmid and comprising a gene or an open reading frame. They were cloned from different angiosperms as indicated in Table 2. The second set of probes was composed of five 30- to 40-kb maize mt DNA cloned in cosmids. As the exact relationship between the two sets of probes was not known, they were considered as representing two independent experiments.

Genetic distance computation and multivariate analysis. A single accession was kept to represent all accessions with the same restriction patterns. Genetic distance was considered as $D = -\log F$.

Table 2. List of mitochondrial DNA probes utilized

Gene or ORF	Insert size (Kb)	Reference	Number of bands	
Plasmid probes			per plant	total
CO II (pea)	1.9	A. Morikami & K. Nakamura, pers. comm.	2	2
CO III (*Oenothera*)	1.1	Hiesel et al., 1987	1	6
CO I (*Oenothera*)	2.6	Hiesel et al., 1987	2 or 3	13
ATP-9 (*Petunia*)	3.5	Rothenberg & Hanson, 1987	1 or 2	8
ATP-9 (*Oenothera*)	6.3	Schuster & Brennicke, 1989	1	1
ATP-6 (*Oenothera*)	4.2	Schuster & Brennicke, 1987	1 or 2	13
ATP-A (pea)	1.5	Morikami & Nakamura, 1987	1	5
26S rRNA (wheat)	5.1	Falconet et al., 1985	2	3
18S and 5S rRNA (wheat)	3.2	Falconet et al., 1984	1 to 4	11
COB (wheat)	5.6	Boer et al., 1985	1 or 2	8
NAD3 (*Petunia*)	0.6	Rasmussen & Hanson 1989	1	4
RPS12 (*Petunia*)	0.2	Hanson et al., 1989	1	4
ORF-25 (*Petunia*)	1.1	Folkerts & Hanson, 1989	2 to 4	10
NAD-1 (watermelon)	4.5	Stern et al., 1986	1	2
		Total number of bands with plasmid probes		86
Cosmid clones (Maize)	30 to 40			
9-3B7		Lonsdale et al., 1983	3 to 4	5
9-3H10		Lonsdale et al., 1983	7 to 9	16
2c70		Stern et al., 1984	5 to 8	18
9-1D10		Stern & Lonsdale 1982	3 to 6	16
8-3F12 (contains the 26S rRNA gene)		D. Stern & D. Lonsdale, pers. com.	2 to 5	11
		Total number of bands with cosmid probes		66

F is the ratio of common bands over total number of bands in the comparison of all bands observed in two individual plants and with a set of probes. Data Desk program for Macintosh computers (Odesta Corporation) was used to compute complete linkage dendrograms and principal component analysis (with covariances option) from the square matrix of distances between distinguishable accessions.

Results

Restriction fragment patterns observed

The number of bands with autoradiographs of major intensity observed with the various probes and an *Eco*RI digestion was fairly constant across all accessions of genus *Oryza*, and across a number of additional accessions including most genera in the tribe Oryzeae (listed in Zhang & Second, 1989), plus barley, oat, wheat, and sorghum. One exception, the *O. meyeriana* complex, had a higher number of bands with most probes. Bands with autoradiographs of minor intensity were ignored.

For plasmid probes, the number of bands varied between 1 and 5 for a given plant, with a total of 86 bands observed in the section *Oryza* plus *O. brachyantha*. The total number of bands observed for a given plant as given in Table 1 varied little in the section *Oryza*: from 18 in *O. rufipogon* to 23 in CD genome species, with an average of 21.5. It was approximately the same in other species or genera analyzed, except in *O. meyeriana* and *O. granulata* which had a mean of 34 bands. As an example, Fig. 1 shows the various patterns observed with the probe *cox*3.

As expected, the patterns observed with cosmid probes were more complex. In section *Oryza* plus *O. brachyantha*, the number of main bands varied between three to eight for a given plant, with a total of 66 bands. No apparent duplicate polymorphic band appeared between the partly overlapping probes 9-3B7 and 9-3H10 (Lonsdale et al., 1983). Figure 2 shows as an example various patterns observed with the probe 9-1D10.

Check for cross hybridization with chloroplast DNA. Bands that were assumed to correspond to

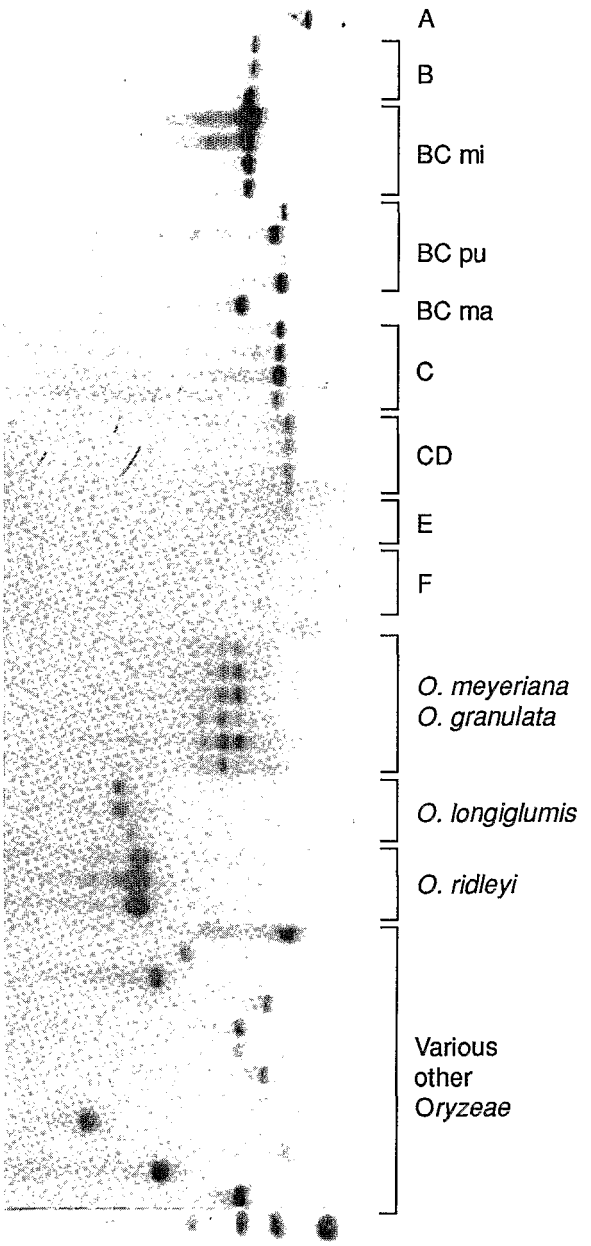

Fig. 1. Main patterns of hybridization with the plasmid probe cox3 as observed in this study. The genomes or species corresponding are indicated with the symbols used in Table 1. At the *Hind*III digested lambda phage DNA as molecular size marker (migration was from right to left).

contaminant mt DNA could be seen after hybridization with mt DNA probes of membranes blotted with purified restricted cp DNA. When polymorphic, however, these bands could never be matched

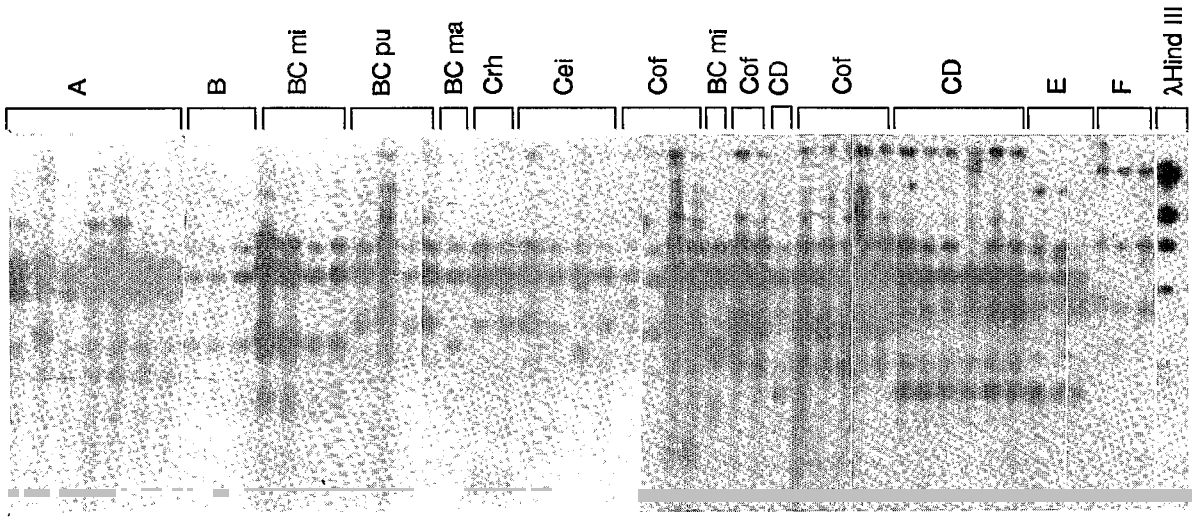

Fig. 2. Main patterns of hybridization with the cosmid probe 9-1D10 observed in this study. The genomes or species corresponding are indicated with the symbols used in Table 1. On the right, a *Hind*III digested lambda phage DNA as molecular size marker.

to any of cp DNA band systematically observed from the same membranes.

Variation within and between species.

Section Oryza and O. brachyantha

Two matrices of genetic distances between accessions that could be distinguished were generated independently from bands observed with plasmid and with cosmid probes, respectively. From each matrix, a dendrogram was generated (showing the relationships among accessions (Fig. 3A-a and 3B-a).

A striking result is that variation within species was low, with many of the accessions indistinguishable or differing only in a small number of bands. In contrast, the distances between genomes were large.

O. brachyantha, with the F genome, appeared as expected, distantly related to the section *Oryza*, while the relationships among genomes of that section appeared to be sometimes contradictory in the two dendrograms. Consequently, the matrices of distances were further analyzed by principal component analysis (PCA), without *O. brachyantha*, as shown in Fig. 3A-b and 3B-b. The relative position of the B genome in the analysis from the plasmid and cosmid probing differed as either the closest or the more distant from the A genome. A consensus, however, from the two sets of data was that the A genome (group Sativa) is distinct from other genomes in the section, while B to E genomes form another natural but more heterogeneous group (group Latifolia).

The observed polymorphism within group Sativa seemed too small to warrant further detailed analysis (given the small number of accessions in each species) but was larger in the group Latifolia. Another PCA was performed on the matrix of distances between accessions of group Latifolia only, as shown in Fig. 3A-c and 3B-c. The fact that in the dendrograms the CD genome clustered either with the E genome (plasmid probes) or with the C genome (cosmid probes) while the E genome was found in cluster either with the B or with the CD genomes appeared to be due to a continum on the main axis of variation with a sequence B, E, CD, and C for both sets of probes.

The actual distances between genomes or species as found in the present analysis are given in Table 3 as the average of the pair-wise distances between distinguishable accessions, when there was more than one distinct accession per species. The three diploid genomes B, C, and E in the group Latifolia, appeared well distinguished from each other with approximately equal distances. *O. rhizomatis* (earlier named *O. collina*, Vaughan, 1990) showed the highest within-genome divergence.

The allotetraploid genomes showed interesting relationships with their diploid counterparts. Two different patterns of mt DNA were found in the

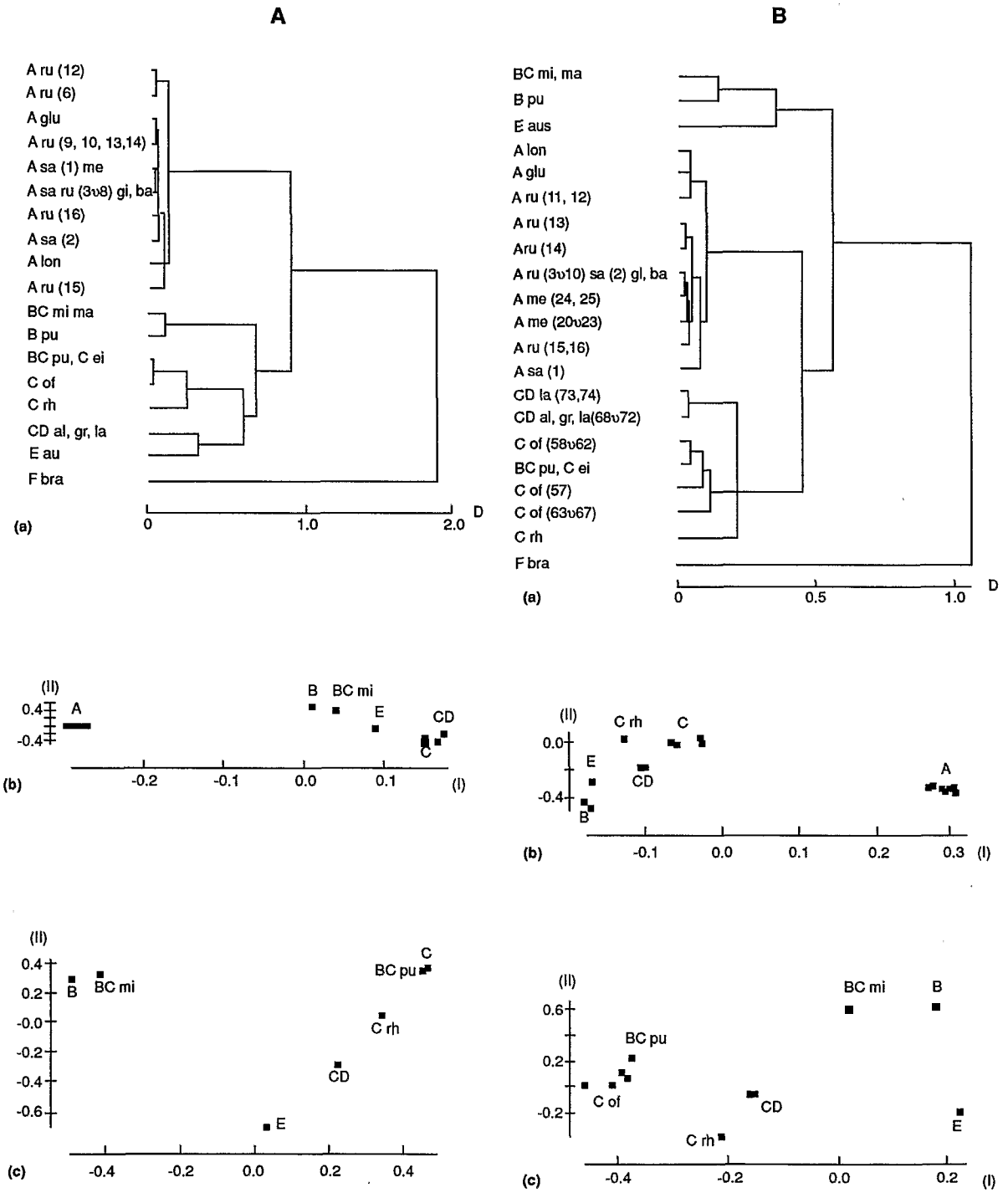

Fig. 3. Multivariate analysis of the matrix of distances based on RFLP between accessions of the section Oryza plus *O. brachyantha*, from plasmid probes (A) or cosmid probes (B). (a): a complete linkage dendrogram between all accessions. The value of distance (D) is indicated. Symbols of genomes and species are as in Table 1. When ambiguous, the accession numbers given in Table 1 are indicated in parenthesis. (b) and (c): Principal component analysis of the matrix of distances for all accessions except *O. brachyantha* (b), or distinct accessions of the group Latifolia only (c). The planes are defined by the first (I) and second (II) eigenvectors. The relative scale of the two axes is according to the relative proportion of variance extracted by each axis (more than 85% total variation is extracted by every plane). The variance of the projection on the two main axes is indicated.

Table 3. Genetic distances between genomes and sub-genomes derived from mt DNA RFLP (on and above the diagonal, average distances from plasmid and cosmid probes. Below the diagonal, distances from plasmid probes only. Genome species symbols as in Table 1)

	A	B	BCmi BCma	Cof	Cei BCpu	Crh	CD	E	F
A	0.04	0.72	0.69	0.70	0.68	0.78	0.73	0.86	1.55
B	0.69	0.00	0.13	0.76	0.73	0.79	0.66	0.73	1.39
BCmi, BCma	0.69	0.11	0.00	0.63	0.60	0.64	0.59	0.77	1.43
Cof	0.96	0.96	0.85	0.06	0.05	0.30	0.38	0.84	1.50
Cei, BCpu	0.94	0.94	0.82	0.02	0.00	0.23	0.33	0.77	1.42
Crh	0.91	0.92	0.80	0.34	0.31	0.00	0.44	0.66	1.41
CD	0.98	0.87	0.76	0.47	0.45	0.58	0.02	0.38	1.47
E	0.94	0.94	0.94	0.99	0.97	0.62	0.38	0.00	1.42
F	1.84	1.84	1.84	1.89	1.87	1.84	1.91	1.87	0.00

BC genome species. *O. minuta* and *O. malampuzhaensis* shared the same pattern, very close to that of the B genome (*O. punctata* 2n) for both types of probes. For plasmid probes the only differences are as follows: the larger fragment hybridizing with *atp*6 was specific to the allotetraploids and the only fragment hybridizing with *atp*9 was the same in the BC and the C genomes, longer than in the B genome. *O. punctata* (4n) had exactly the same patterns as those in *O. eichingeri* with C genome. Compared with *O. officinalis* for plasmid probes, *O. punctata* (4n) and *O. eichingeri* lacked only a short fragment specific to *O. officinalis* in *atp*9.

In the CD genome species, surprisingly, mt DNA was approximately equidistant between those of the C and the E genomes, except for one band otherwise specific to the B genome (and related BC genome) and one band found only in the CD genome. The exact relationships of individual restriction patterns of mt DNA of the CD genome for plasmid probes is as follows: same bands as in the C genome: *atp*6 16 and 1.6 kb, *atp*9 16 kb, *cox*1 19 and 3.3 kb; same bands as in the E genome: *atp*A 2.3 kb, *cox*3 17 kb, *cob* 2.0 kb, 18 + 5s 7.5 and 5.3 kb, *nad*3 13 kb, *orf*25 17 kb; same band as in the B genome: *orf*25 6.3 kb; unique band: *orf*25 1.5 kb.

O. meyeriana and O. ridleyi complex

No variation was observed between accessions of the *O. meyeriana* complex. A small interspecific polymorphism appeared in the *O. ridleyi* complex between *O. ridleyi* and *O. longiglumis* with two bands differing, one each in *orf*25 and *cox*3.

There was a very large polymorphism, however, between the *O. meyeriana* and the *O. ridleyi* complex, and between them and all other species of the genus *Oryza*, including *O. brachyantha*. All bands were different, except one in *atp*A common to the *O. ridleyi* complex and *O. brachyantha*, and one in each of the two following probes: *atp*9 from *Oenothera*, 26S and 18 + 5S. These last common bands were actually monomorphic across all grass species surveyed. The large divergence of the *O. meyeriana* and *O. ridleyi* complex, as well as that of *O. brachyantha*, in the genus *Oryza*, is therefore out of the range that can be relatively quantified with the present data.

Variation among cultivated rice. The same representative collection of *O. sativa* used by Wang & Tanksley (1989) plus two varieties used by McCouch et al. (1988) and 15 *O. glaberrima* accessions were studied. Only four probes were hybridized: *atp*6, *co*1, *cob*, and *orf*25. As an example, the patterns seen with the probes *atp*6 and *cob* are shown in Fig. 4. A total of 15 polymorphic fragments (including nine that appeared clearly in cultivated rice but were not seen or were weak in the observed wild species) and four monomorphic fragments could be scored. They represented a total of nine different patterns, as shown in Table 4.

Figure 5 shows the relationships among these nine mt DNA patterns in terms of distance. As is usual in cultivated rice, three groups are found: *O. glaberrima* has a distinct unique pattern (G) while the patterns observed in *O. sativa* fall in two groups with three and five patterns, respect-

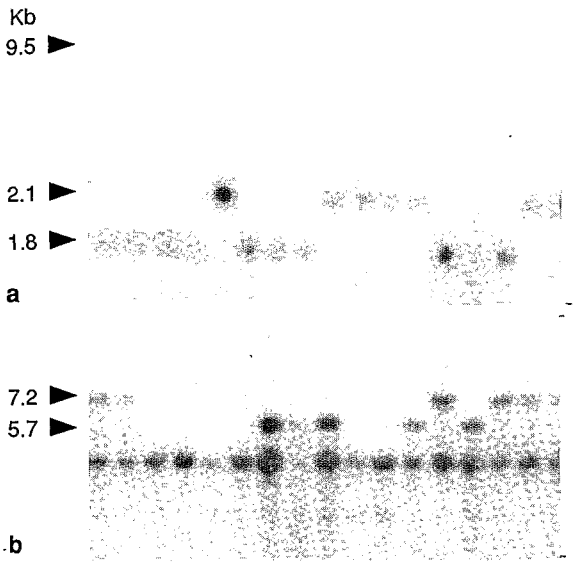

Figure 4. Hybridization patterns seen with the probes *atp6* (a) and *cob* (b) in cultivated rice. The molecular weight of the variable fragments is indicated.

ively. The repartition of the mt DNA patterns among *O. sativa* cultivars is shown in Table 5. To show the relationships between single copy n DNA RFLP, isozyme groups of varieties (I to VI, Glaszmann, 1987), and cytoplasmic DNA RFLP, the *O. sativa* cultivars in Table 5 are sorted according to their order on the first axis of variation (76% of total variation) of a PCA (as used in Fig. 3) of their matrix of distances from RFLP fragments observed with 57 nuclear genomic probes (data as in Wang & Tanksley, 1989 and additional unpublished data). This sorting of varieties according to n DNA RFLP variation appears to be clearly correlated with the isozyme groups and this corresponds to the classical differentiation seen in *O. sativa* with two subspecies: *japonica* (isozyme group VI) and *indica* (group I), and intermediates (groups II, III, IV and V).

It appears that each of the two groups of *O. sativa* mt DNA patterns of Fig. 5 includes, respectively, the patterns most common in *indica* and *japonica* varieties. For that reason, these groups were associated with a *japonica* (J) and an *indica* (I) type of pattern. With this convention, it appears that the distribution of "I" and "J" mt DNA patterns among *O. sativa* cultivars parallels that of the cp DNA patterns, when known, as shown in Table 5. Isozyme group VI cultivars presents the *japonica* type of cytoplasmic DNA patterns only, while isozyme group I cultivars presents both *indica* and *japonica* patterns. In intermediate cultivars, both *indica* and *japonica* types of cytoplasmic DNA patterns are also found, but the *japonica* pattern is more frequent.

As seen in Fig. 5, mt DNA patterns of the intermediate cultivars (I3, J2, J3, J4 and J5) are not intermediate in terms of distance but seem on the contrary to have accumulated differences compared with the "typical" *indica* or *japonica* patterns I1, I2, and J1.

Table 4. Polymorphic mt DNA bands observed in cultivated rice with four probes and the frequency of the various patterns observed. I, J and G characterise patterns most frequent or observed only in *indica*, *japonica* and *glaberrima*, respectively

Probes:		ATP-6			CO-I			COB		ORF-25						
Band MW (Kb):		1.8	2.1	9.5	1.7	2.5	6	6	7	2.3	2.7	4.2	5.7	6.3	11	19
Patterns	Frequency	Y(a)	Y	Y	Y	N	N	Y	N	N	N	N	N	N	N	Y
I1	17	0	1	1	0	1	1	1	0	0	1	0	1	0	1	0
I2	5	0	1	1	0	0	1	1	0	0	1	0	1	0	1	0
I3	1	1	0	1	0	1	1	1	0	0	1	0	1	0	1	0
J1	18	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0
J2	12	1	0	0	1	1	0	0	1	1	0	0	1	0	1	0
J3	9	0	1	0	1	1	0	0	0	1	0	0	1	0	1	0
J4	5	0	1	0	1	0	0	0	0	1	0	0	1	0	1	0
J2	2	1	1	0	1	1	0	0	1	1	0	0	1	0	0	0
G	15	0	1	1	1	0	0	0	0	0	0	1	0	1	0	1

(a) Y/N indicates the band was/was not observed in the wild species.

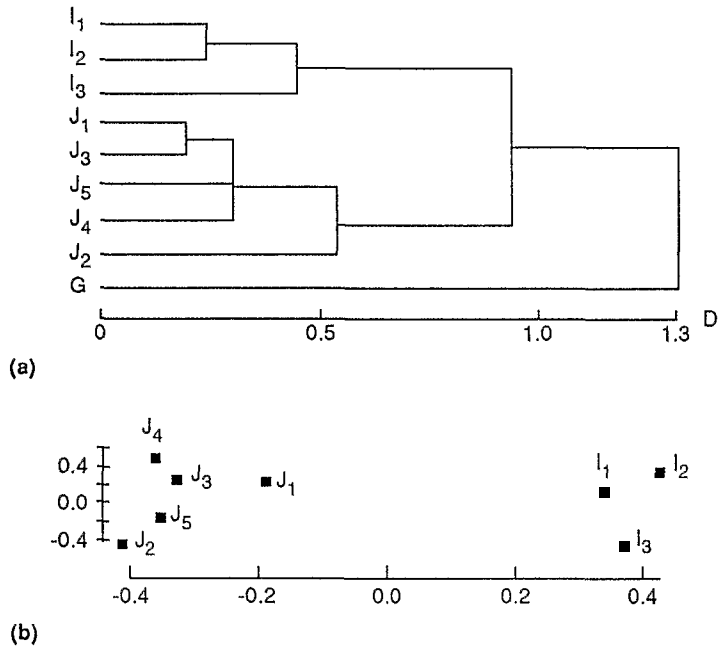

Figure 5. Multivariate analysis of the matrix of distances between the 9 mt-DNA patterns observed in cultivated rice, symbolized as in table 4. (a): a complete linkage dendrogram. The scale of distances (D) is indicated. (b): a principal component analysis of patterns observed in *O. sativa*. The plane is defined by the first (I) and second (II) eigenvectors (83 and 12% of total variation, respectively). The variance of the projection on the two main axes is indicated.

The pattern J5 is worth particular mention as it presents both the 1.8 and 2.1 kb *atp*6 bands and is observed only in two isozyme group II varieties: Chinsurah Boro II known to induce cytoplasmic male sterility in crosses with some *japonica* varieties, and FR13A—also from East India—one of the most flood-tolerant varieties.

The extent of RFLP between one representative each from the *indica* and *japonica* groups of mt DNA patterns was further studied with the two parental varieties (with the J4 and I1 patterns) used in McCouch et al. (1988) digested with nine different restriction enzymes and probed with 16 different clones, as shown in Table 6. The degree of polymorphism is impressive as only *atp*9 from *Oenothera* and cosmid probe 2C70 did not detect any polymorphism. Three probes detected polymorphism with a single enzyme (probably cases of restriction site mutation) and 12 probes detected polymorphism with two and up to eight enzymes (thus likely to correspond in a majority of cases to addition/deletion or inversion type of mutations). The high degree of polymorphism detected in cultivated rice contrasted with the relatively low polymorphism generally detected within species or even genomes in wild species.

Discussion

The technique. A clear advantage of hybridizing mt DNA probes on total DNA versus purifying mt DNA is to allow study from individual plants representing a broad spectrum of genetic variation.

Mitochondrial DNA, including that from rice, is known to contain cp DNA sequences (Stern & Lonsdale, 1982; Newton, 1988; Moon et al., 1988). There is a possibility that mt DNA probes cross-hybridize with cp DNA. In particular, among the cosmid probes utilized, some are known to cross-hybridize with cp DNA. (Lonsdale et al., 1983). However, none of the polymorphic bands we observed could be related to a cp DNA band.

It is also known that mt DNA contains sequences that cross-hybridize with single copy n DNA (Newton, 1988; Kadowaki et al., 1990), and plasmid-like mt DNA has homologous sequences

Table 5. Distribution of observed mitochondrial DNA patterns ("MT") among a representative set of *O. sativa* cultivars previously characterized for nuclear DNA RFLP ("N": values are the coordinates, given in ascending order, of the cultivars on the first axis representing 76% of total variation of a Principal Component Analysis of n DNA RFLP obtained with 57 single copy probes—Wang and Tanksley 1989 and unpublished data), isozyme varietal groups ("VG": as given in Wang & Tanksley, 1989) and chloroplast DNA RFLP patterns ("CP": J and I stand for "*japonica*" and "*indica*" groups of patterns, respectively with the same conventions as for mt DNA. Numbers or e1 and e4 refer to pattern symbols used in Ishii 1991 or Dally 1988, respectively. Parenthesis indicate analysis was incomplete, or the same variety but a different accession was analysed. Dash indicates unknown pattern)

Name (IRRI Acc.)	N	VG	CP	MT
Cicih Beton (43372)	−0.175	VI	J1	J1
IRAT 13 (28508)	−0.173	VI	—	J1
Moroberekan (12048)	−0.172	VI	—	J1
Dam (23710)	−0.167	VI	—	J1
Trembese (43675)	−0.166	VI	J1	J1
Gotak Gatik (43397)	−0.165	VI	—	J1
Binulawan (26872)	−0.162	VI	—	J1
Hawm Om (23729)	−0.160	VI	Je4	J1
Azucena (328)	−0.156	VI	J1	J1
OS 4 (11335)	−0.155	VI	—	J1
Aichi Asahi (40252)	−0.142	VI	—	J1
Beonjo (55457)	−0.141	VI	J1	J1
Haifugoya (17054)	−0.136	VI	J1	J1
Shan Kiu Ku (1154)	−0.124	VI	—	J1
Ta Hung Ku (1107)	−0.102	VI	J1	J1
Rayada 16-04 (27590)	−0.087	IV	I10	J3
Rayada 16-05 (27591)	−0.083	IV	Je1	J3
Kaukkyi Ani (33188)	−0.082	V	J1	J2
Rayada 16-02 (27588)	−0.077	IV	JE1	J3
Rayada 16-06 (27592)	−0.075	IV	—	J3
Dom-Sofid (2880)	−0.063	V	—	J2
Rayada 16-03 (27589)	−0.061	IV	—	J3
Chhoti Mashino (58931)	−0.055	O	—	J2
Basmati 370 (6426)	−0.052	V	J1	J2
Gompa 6 (12898)	−0.049	O	—	J4
Matia Aman 53-13 (37764)	−0.046	IV	—	I3
Batak 640 (29259)	−0.038	O	—	J2
Heuksaekdo (55535)	0.002	O	—	J3
Aswina (26289)	0.020	III	—	J2
PTB 29 (6434)	0.027	O	—	J4
ARC 13839 (42469)	0.029	V	—	J3
Jhona (6307)	0.042	O	—	J4
Chinsurah Boro II (11484)	0.046	II	Je1	J5
Dular (32561)	0.047	II	I3	I1
Warrangal 1240 (13742)	0.052	O	—	I2
T 26 (46768)	0.055	V	—	J2
Pankhari 203 (5999)	0.057	V	—	J2
Goia (49189)	0.072	III	—	J2, J1
DA 10 (6245)	0.076	II	—	I1
FR 13A (6144)	0.082	II	—	J5
Laki (26389)	0.095	III	—	I1, J2
Guan-Yin-tsan (51300)	0.105	I	I3	I2
Tetep (32576)	0.109	I	—	I1
Khao Dawk Mali (27748)	0.113	I	—	I1
JC 117 (9179)	0.114	I	—	I1
Bhadoia 233 (6541)	0.118	III	J1	J2
Bamoia 341 (6538)	0.119	III	I3	I1
DA 9 (5854)	0.119	I	—	J2
Ilis Air (43400)	0.130	I	I3	I1
IR 8 (10320)	0.132	I	—	I1
Leuang Pratew (27762)	0.133	I	(I3)	I1
ASD 1 (6267)	0.134	I	(I3)	I1
Carreon (32575)	0.134	I	—	I1
Sinna Sithina Kali (51064)	0.138	I	I3	I2
Nam Sa-gui 19 (11462)	0.139	I	—	J4
S 624 (8896)	0.140	I	J1	J2
Kaw Luyowng (27716)	0.146	I	—	I1
PTB 9 (6274)	0.146	I	J1	J2
IR 5 (10321)	0.154	I	(I3)	I1
Patik (43530)	0.157	I	J11	J4
Intan (4230)	0.158	I	—	I1
Cere Air (43369)	0.159	I	I3	I1
Peta (32571)	0.161	I	—	I1
Ai chiao Hong (51250)	0.163	I	(I3)	I2
Chau (56036)	0.167	I	I3	I2

in n DNA (Shikanai et al., 1989; Sakamoto et al., 1991). We used Southern filters that were largely exhausted for their capacity to cross-hybridize with single or low copy number n DNA in the experimental conditions utilized. Moreover, we did not score faint hybridization bands. That the patterns we scored do not correspond to any n DNA fragment is further confirmed by the fact that allotetraploid species did not show an increase in number of fragments compared with the diploid species. Also, in cultivated rice, the mt DNA patterns are clearly correlated with the cp DNA, but not with the n DNA patterns.

Plasmid probes probably hybridize to more than 40 kb scattered on the total rice mt DNA molecule (*atp*A, *atp*9, *cob*, *cox*1, *cox*2, 26S and 18S are known to be scattered along the mt DNA molecule (Hirai et al., 1991)), and cosmid probes probably hybridize to more than 150 kb of the mt DNA molecule. In total, more than one-fourth of the estimated 526 kb rice mt DNA have probably been probed. Our probing with both plasmid and cosmid clones represents two independent experiments and protects against large statistical deviation in the computation of distances.

Evolution of mitochondrial genome. It is known that mt DNA of higher plants evolves slowly in

Table 6. Survey of polymorphism between an *indica* and a *japonica* variety in hybridization patterns with 16 mt DNA probes for nine restriction enzymes

Restr. enz.	EcoRI	EcoRV	DraI	HindIII	ScaI	XbaI	BamHI	BglII	MspI
Probes									
CO II	M (a)	M	P	M	M	M	M	M	M
CO III	M	M	M	M	M	M	P	M	M
CO I	P	P	M	P	P	P	P	P	M
ATP-9oe	M	M	M	M	M	M	M	M	M
ATP-6	P	P	P	P	P	M	P	M	M
ATP-A	M	P	M	M	M	M	M	M	M
26S rRNA	M	M	M	M	P	P	M	M	M
18s + 5S rRNA	P	P	P	M	M	M	M	M	M
NAD3	M	M	P	M	M	P	M	M	M
ORF-25	P	M	P	P	M	M	P	M	P
NAD-1	P	P	P	P	P	M	P	P	P
9-3B7	M	P	M	P	P	M	P	P	M
9-3H10	M	P	P	P	P	P	P	P	P
2c70	M	M	M	M	M	M	M	M	M
9-1D10	P	P	P	P	M	P	P	P	M
8-3F12	P	M	M	P	M	M	P	P	P
Total	7P	8P	8P	8P	6P	5P	9P	6P	4P

(a) M: monomorphic patterns, P: polymorphic patterns

sequence but rapidly in structure (Newton, 1988; Palmer & Herbon, 1988). That mt DNA evolves rapidly at RFLP level in rice compared with cp DNA is indicated by the fact that most mt DNA restriction fragments differ in size between the diploid genomes, even in the section *Oryza*, while most cp DNA restriction fragments between genomes and even within genus *Oryza* are similar. If we assume that the DNA sequence is even more conserved in plant mitochondria than in the chloroplast (Wolfe et al., 1987), it is clear that a very large proportion of RFLP observed must be due to structural variation.

A case of profound alteration in structure of mt genome was seen in the *O. meyeriana* complex, which showed a significantly higher number of restriction fragments than did other species. This could indicate a higher proportion of duplicated sequences in its genome or the coexistence in high proportion of several altered mt DNA molecules. However, as in the other species, there was a remarkable homogeneity in the number of main restriction fragments across this complex.

Phylogenetic relationships in genus Oryza. Information on mt DNA RFLP among wild species, was obtained mostly between genomes. Results clearly reinforced the natural classification of genus *Oryza* and the recognition of genomes. This indicates that, in general, n DNA, cp DNA, and mt DNA have evolved parallel to one another.

For BC genome allotetraploids, the same maternal parents as from cp DNA studies (Dally & Second, 1990) are indicated from mt DNA variation, with at least two different allotetraploidization events, one leading to tetraploid *O. punctata* with a cytoplasm from the C genome and one leading to *O. malampuzhaensis* and *O. minuta* with a cytoplasm from the B genome. The observed difference between presumed maternal and allotetraploid mt DNA is small or null and is compatible with a recent origin of these allotetraploids. However, there was a slight increase in similarity of the mt DNA of BC allotetraploids and of the mt DNA of their presumed paternal parents, compared with the level of similarity between the maternal and paternal presumed parents.

For the CD genome allotetraploids, a relationship unsuspected from the results at cp DNA level was seen. At cp DNA level, the CD genome species have a close relation with those in the C genome, which thus represents the presumed maternal parent (Dally & Second, 1990). At mt DNA level, in contrast, CD genome species clearly show an affinity with both the C and the E genome species.

An affinity of CD genome with E genome was shown recently at n DNA RFLP level (Wang et al., 1991), this sustains observations made earlier at cytogenetic (Katayama, 1982) and isozyme (Second, 1985) levels. However, assuming mt DNA is strictly maternally inherited along with cp DNA, no particular relationship should be expected with the E genome. The same distance between CD and E genomes should be expected between the C and E genomes, but that is clearly not the case (0.38 *vs* 0.99 for plasmid probes and 0.38 *vs* 0.84 on average between plasmid and cosmid probes, as shown in Table 3).

While, to our knowledge, no case of recombination or specific rearrangement in mt DNA induced by nuclear DNA has been reported in nature, there is a growing body of evidence that mt DNA recombines readily in somatic hybrids or cybrids obtained *in vitro*. The process of organelle sorting and mt DNA rearrangements, including intergenomic recombination, appears to be under selection pressure and in interaction with the nucleus. Intergenomic recombination is characterized by restriction patterns comprising fragments from both parents as well as hybrid-specific mt DNA fragments (Belliard et al., 1979; Thanh et al., 1988; Rothenberg et al., 1985; Vedel et al., 1986; Asahi et al., 1988; Landgren & Glimelius, 1990; Wachocki et al., 1991).

The observed pattern of variation of mt DNA in the CD genome is reminiscent of what is observed in some somatic hybrid derivatives. We feel our data represent indirect evidence of natural interspecific hybridization at mt DNA level. To our knowledge, this would be the first case reported in nature. Possibly, biparental inheritance of mitochondria is more likely to occur in the course of an interspecific hybridization, which is undoubtedly at the origin of the CD genome. Direct selection for paternal inheritance of chloroplast has been reported in sexual progeny of *Nicotiana* (Avni & Edelman, 1991).

As an alternative to interspecific recombination to interpret an increased similarity of the mt DNA of allotetraploids with their presumed paternal parent, nuclear-controlled rearrangement of mt genome could be invoked. This could be an acceptable explanation for the limited increase in similarity observed in BC genome allotetraploids. It would take complex rearrangements, however, to explain the patterns observed in the CD genome species. Nuclear influence on mitochondrial genome structure as compared between normal and alloplasmic *Nicotiana* materials was found (Håkanson & Glimelius, 1991) but most of it was quantitative. On the other hand, stability of the mitochondrial genome in the allotetraploids *Nicotiana tabacum* and *Brassica* spp. compared with that of their maternal parents has been documented (Bland et al., 1985; Palmer, 1988). Sequencing of the less conserved portions of genes used as probes from the various genomes in the *Oryza* section should shed light on this problem.

Variation observed in cultivated rice. A surprising result is that mt DNA in cultivated rice shows both a relatively higher variation than in the wild species and fragments not observed in wild species, at least not as major bands. This result can be correlated with an observation made by Ishii & Tsunewaki (1989) who found quite different purified mt DNA restriction patterns between some *O. sativa* cultivars and one wild strain of Asian *O. rufipogon* with the same cp DNA restriction pattern.

The nine observed patterns clearly grouped according to the classical distinction of two cultivated species *O. sativa* and *O. glaberrima* and two main subspecies *indica* and *japonica* (including javanica type) in *O. sativa*. Only *indica* and intermediate *indica/japonica* cultivars showed either *japonica* or *indica* mt DNA patterns with several variants each, while *O. glaberrima* and the *japonica* subspecies of *O. sativa* both had a single mt DNA pattern. Assuming domestication of *japonica* and *indica* cultivars started in different wild forms, each with a small intraspecific variation, and was followed by reciprocal introgression (Second, 1982), this might be an indication that the increased diversity in *indica* and intermediate varieties accompanied the introgression of the *japonica* pattern.

There was a clear correlation between the mt DNA and cp DNA patterns in terms of their classification in "*indica*" or "*japonica*". Closely related cultivars can have either a japonica or an indica type of organelle DNA. There was one possible case (acc 27590, in Table 5) of a variety with cp DNA of an indica type with two unique mutations (pattern 10 in Ishii, 1991 and mt DNA of the japonica type. Intra accession diversity as

seen in acc. 26389 for mt DNA (Table 5) is likely to explain this case.

How the observed polymorphism could be associated with the existence of plasmid-like mt DNA in cultivated rice (Kadowaki et al., 1988b) requires further studies. We may note, however, that *japonica* subspecies generally lack both plasmids and polymorphism.

Relation with cytoplasmic male sterility. Comparative studies of normal and male sterility inducing cytoplasms for RFLP in mt DNA have shown a number of differences, particularly at the level of a *Pst*1 fragment including *atp*6 and *cob* genes. The presence of a chimeric gene containing a portion of the *atp*6 gene was also associated with cytoplasmic male sterility. This polymorphism was thought to have arisen through intra or intermolecular recombination of mt DNA in the course of domestication (Kodowaki et al., 1990). We confirmed the result of RFLP between normal and Chinsurah boro II cytoplasm and showed, in addition, a number of other polymorphisms for the same probes. As discussed earlier, some of these polymorphisms could have arisen in the course of introgression between *indica* and *japonica* subspecies. It seems advisable to include both *indica* and *japonica* type cytoplasms in the characterization and study of origin of cytoplasmic male sterility.

Rice genetic resources. The contribution of this study in generating knowledge important to assist in the conservation and use of rice genetic resources may be seen at several levels.

The reported observations reinforce the presumed natural classification of *Oryza* and help to select a small number of accessions for further genetic and agronomic evaluation. The interpretation points to a likely origin of the CD genome mitochondrial DNA from interspecific recombination and gives strength to the hypothesis of a recent origin of the CD genome. This, in turn, leads to a revision of the systematics of *Oryza* from which most wild species are assumed to have evolved from a few ancestral species, following indirect human intervention which favored gene flow through introgressive hybridization and allotetraploidization across pre-existing geographic barriers to plant migration (Second, 1991).

A new variation detected in cultivated rice at mt DNA level shows that cultivated forms have also accumulated molecular variation since domestication. As this development seems related to the introgression of a *japonica* cytoplasm along an *indica* nucleus, it reemphasizes the importance of genetic exchange between the *indica* and *japonica* subspecies in the domestication of rice and thus in rice breeding.

Acknowledgements

The Institut Français de Recherche Scientifique pour le Développement en Coopération (Orstom) and the International Rice Research Institute (IRRI) provided the plant material. Orstom and the Rockefeller Foundation gave their financial support. Drs T. Ishii, M. Hanson and D. Stern made the clones of mitochondrial genes and sequences available to us. Authorizations to use them were given when appropriate by Drs A. Brennicke, M. Gray, M. Lonsdale, F. Quetier and A. Morikami. Dr M. Sugiura provided the chloroplast DNA clones. The RFLP analyses were done in the laboratory of Dr S. Tanksley who also edited the manuscript. Drs M. Jackson, T. Ishii and Mrs G. Argosino also deserve credit for critically reading and editing the manuscript.

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