Studies on the origin of crop species by restriction endonuclease analysis of organellar DNA. I. Phylogenetic relationships among ten cereals revealed by the restriction fragment patterns of chloroplast DNA¹⁾

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ABSTRACT

The phylogenetic relationship of ten crops selected from all the seven tribes of the Gramineae which contain crop species, i.e., wheat, rye and barley from Triticeae, oats from Agrostaeae, finger millet from Chlorideae, foxtail millet from Paniceae, sorghum from Andropogoneae, maize and job's tears from Maydeae, and rice from Oryzeae, were studied by restriction endonuclease analysis of their chloroplast (ct) DNAs. Three enzymes, *Kpn*I, *Pst*I and *Sal*I which are known to give rise to fewer restriction fragments from the cereal ctDNAs than other enzymes were used. For each restriction fragment pattern, the copy number and molecular size of the individual fragments were estimated. From these data, genetic distance ($\hat{p}$) between chloroplast genomes of the ten cereals were calculated using Engels' formula (Engels 1981), based on which their phylogenetic tree was constructed by the UPGMA method of Sneath and Sokal (1973). This phylogenetic tree is in complete agreement with that made from ordinary systematic studies (Tateoka 1957), except for one point, i.e., the genetic distance between the chloroplast genomes of sorghum and maize or job's tears which belong to different tribes is closer than that between maize and job's tears which belong to the same tribe. Thus, the two genera, *Zea* and *Coix* appear to be better placed in separate tribes. The critical $\hat{p}$ value to separate two taxa into different tribes, sub-families and families was about $\hat{p}=0.02$, 0.05 and 0.12, respectively.

1. INTRODUCTION

The agarose gel electrophoresis of restriction endonuclease digests of organellar DNAs has become a new technique in plant genetics (Atchison *et al.* 1976; Bedbrook and Bogorad 1976; Levings and Pring 1976; Vedel *et al.* 1976). In various plant genera, both chloroplast (ct) and mitochondrial (mt) DNAs

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show great diversity in their restriction fragment patterns, and the analysis of these patterns has proved to be a powerful tool to clarify the phylogenetic relationships among the species in several taxa, such as *Triticum* and *Aegilops* (Vedel *et al.* 1978; Ogihara and Tsunewaki 1982; Terachi *et al.* 1984), *Zea* (Timothy *et al.* 1979), *Oenothera* subsection *Euoenothera* (Gorden *et al.* 1982), *Nicotiana* (Kung *et al.* 1982), *Lycopersicon* (Palmer and Zamir 1982), *Coffea* (Berthou *et al.* 1983), *Brassica* (Palmer *et al.* 1983), legumes (Palmer *et al.* 1983), *Pennisetum* (Clegg *et al.* 1984) and tuberous *Solanum* (Hosaka *et al.* 1984).

Encouraged with these works, we have been studying phylogeny of important crop species by comparative analysis of restriction fragment patterns of organellar DNAs between the crop species and their wild relatives, to shed new light on the origin of the crop species. The family, Gramineae, is divided into five subfamilies and 43 tribes, only seven of which contain cereal crops. The present article, the first report of this series, aims to clarify the phylogenetic relationships among ten crop species belonging to these tribes by the analysis of restriction fragment patterns of their ctDNAs. The results of this study will be compared with those previously obtained by the traditional systematic studies. As reported below, the results have demonstrated that this approach provides useful informations on both the intra- and intertribal phylogenetic relationships.

2. MATERIALS AND METHODS

Ten cereal crops, all belonging to the family, Gramineae, were used in the present investigation. Their lower taxonomic statuses are shown in Table 1. In the foregoing sections, they are called by their common names. Seeds of wheat and job's tears were obtained from the Kyushu Agricultural Experiment Station, Chikugo, and the Chugoku Agricultural Experiment Station, Fukuyama, respectively. Rice seeds were a gift from the Agricultural Research Center, Ministry of Agriculture, Forestry and Fishery, Tsukuba. All other materials were derived from the stock collection of our laboratory.

Seeds of all cereals, except rice, were sown in wooden flats which were kept in a greenhouse or outdoors depending upon the season and species to obtain optimal growth. Rice seeds were sown in flats immersed in water, which were kept outdoors during summer. When seedlings reached a height of about 10 cm, sometimes more to compensate the shortage of seeds, their leaves were collected and used for chloroplast isolation. The regenerated leaves were also used for the same purpose. The plants were kept in darkness for 24 h prior to leaf collection.

The isolation of intact chloroplasts, the preparation of ctDNA from the isolated chloroplasts, and the analysis of restriction fragment patterns of

Table 1. Taxonomic statuses of ten Gramineae crops used in the present investigation

Subfamily	Tribe	Genus	Species	Common name	Lower taxon
Pooideae	Triticeae	<i>Triticum</i>	<i>T. aestivum</i>	wheat	cv. Shirogane-komugi
"	"	<i>Secale</i>	<i>S. cereale</i>	rye	—
"	"	<i>Hordeum</i>	<i>H. vulgare</i>	barley	cv. Goshikoku
"	Agrosteae	<i>Avena</i>	<i>A. barbata</i>	oats	—
Eragrostoideae	Chloridoideae	<i>Eleusine</i>	<i>E. coracana</i>	finger millet	—
Panicoideae	Paniceae	<i>Setaria</i>	<i>S. italica</i>	foxtail millet	—
"	Andropogoneae	<i>Sorghum</i>	<i>S. bicolor</i>	sorghum	—
"	Maydeae	<i>Zea</i>	<i>Z. mays</i>	maize	—
"	"	<i>Coix</i>	<i>C. lacryma-jobi</i>	job's tears	var. <i>major</i> cv. Okayama-zairai
Pharoideae	Oryzeae	<i>Oryza</i>	<i>O. sativa</i>	rice	ssp. <i>japonica</i> cv. Nihon-bare

ctDNAs from all materials, except rice, were carried out according to the procedures of Ogihara and Tsunewaki (1982) and Tsunewaki and Ogihara (1983). Because rice ctDNA prepared by their method was not digested by any restriction endonuclease, we used the liquid nitrogen method for crushing leaves of this crop.

Three restriction endonucleases, *Kpn*I, *Pst*I and *Sal*I, each of which recognizes and restricts a unique six base-pair sequence, were used. The reason for this choice is as follows; as the ten species studied belong to different subfamilies, large variation was expected among their ctDNAs so that we preferred to use the enzymes which give rise to only a small number of ctDNA fragments. The three endonucleases chosen here gave the fewest fragments in wheat and its relatives among the ten enzymes previously employed (Ogihara and Tsunewaki 1982, 1984).

The molecular size of each restriction fragment was estimated by performing three runs with 0.4, 0.8 and 1.2% agarose gel. Size markers used were undigested, *Eco*RI-, *Hind*III-, *Kpn*I- or *Xho*I-digested, and *Eco*RI-*Hind*III double digested lambda phage DNA. Sometimes the *Bam*HI-digested wheat ctDNA was used, because molecular sizes of almost all fragments were reported by Bowman *et al.* (1981, 1983).

The genetic distance, $\hat{p}$, between each pair of chloroplast genomes was estimated according to the method of Engels (1981) from the formula, $\hat{p} = [c - 2(m - k)]/jc$, where c and m are the total numbers of restriction fragments and restriction sites, respectively, in two ctDNA molecules, k is the number of differential restriction sites between the two molecules, and j is the length of a recognition sequence (six for all the three endonucleases). Procedures to obtain m and k from the present data are given in Engels (1981) and Terachi *et al.* (1984).

3. RESULTS AND DISCUSSION

Figs. 1, 2 and 3 show the restriction fragment patterns of ctDNAs of all species except for rice, obtained by the *Kpn*I, *Pst*I and *Sal*I digests, respectively. The three patterns of rice ctDNA obtained by these digests are compared with those of wheat ctDNA in Fig. 4. Estimated molecular weights of all restriction fragments and their copy numbers are given in Table 2 to 4.

The total chloroplast genome size appears to differ among the cereal crops belonging to different subfamilies. Wheat, rye, barley and oats of Pooideae and finger millet of Eragrostoideae had a genome size of about 135 kbp, whereas foxtail millet, maize, job's tears and sorghum of Panicoideae had a ctDNA molecule of about 140 kbp. The chloroplast genome size of rice belonging to Pharoideae was estimated to be about 135 kbp. Bedbrook *et al.* (1979), Bowman *et al.* (1981) and Hirai *et al.* (1985) estimated the chloroplast

Fig. 1.

Fig. 1. *Kpn*I-restriction fragment patterns of ctDNAs of nine cereals: *Kpn*I-digested ctDNA was electrophoresed on 0.8% agarose gel. W: wheat, R: rye, B: barley, O: oats, FM: finger millet, FX: foxtail millet, S: sorghum, J: job's tears, and C: maize. Small fragments (<2.0 kbp) could not be visualized on this gel (ref. Table 2).

Fig. 2.

Fig. 2. *Pst*I-restriction fragment patterns of ctDNAs of nine cereals. (For explanation, see Fig. 1)

genome size of maize, wheat and rice to be 137, 135.2 and 130 kbp, respectively. Vedel *et al.* (1980) reported that the chloroplast genome size of wheat and oats is 81.8 and 81.9×10^6 daltons, respectively, i.e., that the genome size of wheat is similar to that of oats. The chloroplast genome size of wheat and oats we obtained were similar to that estimated by Bowman *et al.* (1981) and Vedel *et al.* (1980), respectively, but that of the maize and rice chloroplast genomes differs from the estimates made in previous works. In the *Sal*I physical map of maize ctDNA drawn by Bedbrook *et al.* (1979), three small *Sal*I fragments (1.1, 0.9 and 0.7 kbp, respectively) are not shown. If they are counted, the maize chloroplast genome is about 140 kbp which is very close to our estimate. The rice chloroplast genome size we estimated is 5 kbp larger than that reported by Hirai *et al.* (1985), which is due to the slightly larger molecular size of each fragment in our estimation than theirs; the restriction fragment patterns we obtained were identical to those they reported. These discrepancies must be solved in future works. So far as our estimates are concerned, the chloroplast genome size of Panicoideae appears to be larger than those of Pooideae, Eragrostoideae and Pharoideae.

From the data presented in Table 2 to 4, the genetic distances ($\hat{p}$ values) between chloroplast genomes of ten cereal crops were estimated using Engels' formula. The results are given in Table 5. Based on these $\hat{p}$ values, a phylogenetic tree was constructed, as shown in Fig. 5, using the UPGMA (un-

Fig. 3.

Fig. 3. *SalI*-restriction fragment patterns of ctDNAs of nine cereals. (For explanation, see Fig. 1)

Fig. 4.

Fig. 4. Restriction fragment patterns of ctDNAs of rice (R) and common wheat (W). *KpnI*, *PstI* and *SalI* refer to the *KpnI*, *PstI* and *SalI* digests, respectively. (*HindIII*-digested lambda DNA is shown as the molecular size marker.)

weighted pair-group mean arithmetic average) method of Sneath and Sokal (1973). This phylogenetic tree for the ten Gramineae crops is in complete agreement with that figured from the conventional systematic studies (Fig. 6), except for one point which will be discussed below.

Wheat, rye and barley, all of which belong to the same tribe, Triticeae, showed a high degree of fragment homology with each other. For example, of 45 fragments identified in the three patterns of wheat ctDNA, 39 (87%) and 32 (71%) had identical counterparts in rye and barley ctDNAs, respectively. Similarly, of the total of 41 fragments in the three patterns of barley ctDNA, 32 (78%) and 30 (73%) had homologous counterparts in wheat and rye ctDNAs, respectively. The genetic distances between the three chloroplast genomes were 0.011–0.021 (Table 5), indicating that they are very closely related. The wheat-rye relationship was closer than the wheat-barley and

Table 2. *Molecular size (kbp) and copy number of individual KpnI-restriction fragments of ctDNAs of ten cereals*

Wheat		Rye		Barley		Oats		Finger millet	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	18.0	1	18.0	1	18.0	1	24.6	1	19.6
1	15.8	2	15.8	2	15.8	1	21.7	1	18.6
1	15.4	1	11.8	1	12.4	1	18.0	1	17.2
1	11.8	3	10.8	1	11.8	1	11.8	1	16.2
3	10.8	1	8.85	2	10.8	2	10.8	1	15.3
1	8.85	1	8.3	1	9.8	1	10.6	2	10.6
1	8.3	1	7.4	1	8.85	1	8.85	1	7.2
1	7.4	1	4.9	1	8.3	1	7.4	1	6.35
1	6.3	1	4.55	1	6.3	1	4.9	1	5.3
1	4.6	3	1.8	2	1.8	2	1.8	1	2.05
3	1.8	2	0.8	2	0.8	2	0.8	2	1.9
2	0.8	1	0.7	1	0.7			2	0.8
1	0.7							1	0.6
18	136.55	18	135.5	16	134.55	14	134.65	16	135.0

Foxtail millet		Sorghum		Maize		Job's tears		Rice	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	19.6	1	21.5	1	21.5	1	21.5	1	19.8
1	17.2	1	20.0	1	20.0	1	20.0	1	18.5
1	15.8	1	16.6	1	16.6	1	17.0	1	17.6
1	14.3	1	15.8	1	15.8	1	15.8	1	14.2
2	10.8	2	11.0	2	11.0	2	11.0	1	11.5
1	10.6	1	10.7	1	10.7	1	10.7	1	11.0
1	8.4	1	8.7	1	8.7	1	8.7	2	10.3
1	7.8	1	8.4	1	8.4	1	8.4	1	8.5
1	7.4	1	7.4	1	7.15	1	6.5	2	3.3
1	5.0	1	5.6	1	5.2	1	5.6	2	2.0
1	2.2	1	2.1	1	2.1	1	2.1	1	1.9
1	2.05	2	0.8	2	0.8	2	0.8	1	1.8
2	1.9	1	0.7	2	0.7	1	0.7		
2	0.8								
1	0.7								
18	138.05	15	141.1	16	141.15	15	140.6	15	136.0

Table 3. *Molecular size (kbp) and copy number of individual PstI-restriction fragments of ctDNAs of ten cereals*

Wheat		Rye		Barley		Oats		Finger millet	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	33.3	1	33.3	2	20.0	1	18.6	1	33.0
1	19.6	1	19.6	1	19.0	1	15.5	1	19.6
1	14.5	1	14.5	1	13.5	1	11.8	1	13.5
1	12.6	1	12.6	1	12.6	1	11.0	1	11.0
1	11.0	1	11.0	1	11.0	1	9.2	1	10.5
2	8.4	2	8.4	1	9.9	3	8.4	1	9.1
1	8.1	1	8.3	2	8.4	1	7.5	2	8.3
1	5.6	1	5.6	1	5.6	1	5.5	1	5.3
1	5.3	1	5.3	1	5.3	1	5.1	1	5.05
1	5.2	1	5.2			1	4.7	1	4.6
1	1.9	1	1.9			1	4.3	1	3.2
1	1.4	1	1.45			2	3.9	1	2.7
						1	3.5	1	0.5
						1	3.1		
						1	1.8		
13	135.3	13	135.55	11	133.7	18	134.6	14	134.65

Foxtail millet		Sorghum		Maize		Job's tears		Rice	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	18.5	1	33.0	1	18.3	1	17.3	1	19.0
1	16.2	1	17.2	1	16.6	1	16.0	1	16.5
1	14.6	1	16.2	1	15.5	1	14.5	1	15.0
1	13.8	1	14.6	1	14.5	1	13.8	1	13.6
1	12.6	1	12.6	1	12.6	1	12.6	1	11.1
1	11.0	1	8.4	1	11.0	1	11.0	1	10.0
1	10.5	3	5.4	1	8.4	1	8.5	2	8.8
3	5.4	1	4.6	3	5.4	3	5.4	1	8.3
1	5.0	1	3.85	1	5.0	1	5.0	1	5.4
1	4.7	1	3.6	1	4.6	1	4.65	1	4.9
1	3.9	2	3.15	1	3.8	1	4.55	1	4.7
2	3.15	1	1.9	2	3.45	1	3.9	1	4.0
1	1.9	1	0.7	1	3.1	1	3.7	1	2.2
1	1.55	1	0.5	2	1.85	2	3.15	1	1.8
1	1.4			1	0.7	1	1.9		
1	0.7			1	0.5	1	0.7		
1	0.5								
20	139.35	17	139.65	20	141.4	19	140.6	15	134.1

Table 4. *Molecular size (kbp) and copy number of individual SalI-restriction fragments of ctDNAs of ten cereals*

Wheat		Rye		Barley		Oats		Finger millet	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	27.2	1	27.2	1	27.2	1	27.2	2	21.8
1	21.8	1	21.8	1	21.8	1	13.6	1	14.5
1	14.2	1	14.2	1	14.2	1	11.8	1	13.6
1	13.6	1	13.6	1	14.0	2	9.2	1	13.2
1	11.8	1	11.8	1	13.6	1	8.6	1	11.8
3	7.2	3	7.2	1	11.8	1	8.0	1	7.3
1	6.8	1	6.8	2	7.2	3	7.2	3	7.0
1	6.2	1	6.3	1	6.2	1	7.0	1	4.1
2	4.5	2	4.5	2	4.5	1	4.95	1	3.25
1	1.2	1	1.2	1	1.2	2	4.5	1	1.1
1	1.1	1	1.1	1	1.1	1	4.1	1	0.8
1	0.8	1	0.8	1	0.8			1	0.7
15	135.3	15	135.4	14	135.3	15	134.25	15	134.95

Foxtail millet		Sorghum		Maize		Job's tears		Rice	
Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size	Copy no.	Mol. size
1	28.0	1	28.0	1	28.0	1	28.0	1	28.0
2	22.5	1	23.1	1	23.1	1	23.1	1	21.4
1	12.8	2	15.5	2	15.5	1	15.5	1	15.8
1	11.8	1	11.8	1	11.8	1	13.2	1	14.8
1	8.9	1	8.9	1	8.9	1	11.8	2	13.2
3	7.3	3	7.3	3	7.3	1	8.9	1	7.6
1	7.0	1	7.0	1	7.0	3	7.3	2	7.4
1	1.95	1	6.8	1	6.7	1	6.85	1	6.9
1	1.1	1	1.1	1	1.1	1	6.7		
1	0.8	1	0.7	1	0.9	1	1.95		
1	0.7			1	0.7	1	1.1		
						1	0.9		
						1	0.7		
14	139.95	13	140.3	14	141.1	15	140.6	10	135.7

rye-barley relationship which supports the fact that wheat and rye are placed in the same subtribe, Triticinae, whereas barley is placed in another subtribe, Hordeinae of Triticeae.

Chloroplast genomes of maize and job's tears which belong to the same

Table 5. Genetic distance ($\hat{p}$) between chloroplast genomes of ten cereals; estimated from the Engels' formula, $\hat{p} = [c - 2(m - k)]/6c$ (ref. the text for c , m and k)

	Wheat	Kye	Barley	Oats	Finger millet	Foxtail millet	Sorghum	Maize	Job's tears
Rye	0.0110								
Barley	0.0155	0.0211							
Oats	0.0471	0.0448	0.0492						
Finger millet	0.0667	0.0641	0.0620	0.0870					
Foxtail millet	0.0636	0.0612	0.0699	0.0724	0.0533				
Sorghum	0.0704	0.0714	0.0736	0.0906	0.0741	0.0292			
Maize	0.0737	0.0747	0.0751	0.0808	0.0754	0.0425	0.0228		
Job's tears	0.0709	0.0719	0.0778	0.0972	0.0851	0.0347	0.0213	0.0286	
Rice	0.1437	0.1250	0.1265	0.1217	0.1092	0.0993	0.1092	0.1087	0.1007

Fig. 5. A phylogenetic tree of the chloroplast genomes for ten cereals; drawn by the UPGMA method (Sneath and Sokal 1973) applied to the matrix of genetic distances shown in Table 5. The critical levels of classifying taxa to separate tribes, subfamilies and families are indicated by the broken lines.

tribe, Maydeae, showed a close relationship with $\hat{p}=0.029$. However, the chloroplast genome of sorghum that belong to another tribe, Andropogoneae, of the same subfamily, was more closely related to those of maize and job's tears ($\hat{p}=0.023$ and 0.021 , respectively), compared to the relationship between the chloroplast genomes of the latter two species. Furthermore, the genetic distance observed between chloroplast genomes of maize and job's tears was the same as that between chloroplast genomes of sorghum (tribe Andropogoneae) and foxtail millet (tribe Paniceae). Therefore, the validity of placing the two genera, *Zea* and *Coix*, into the same tribe becomes questionable. The present ctDNA data are in support of separating these genera into different tribes. The fact that *Zea* and its related genera are of New World origin, whereas *Coix* and its related genera are all of Old World origin supports the present proposition. If this proposition is accepted, the genetic distance, $\hat{p}=0.02$, can be used as the critical value for separating two taxa into different tribes.

The chloroplast genome of oats belonging to the tribe Agrostae showed moderately close relationships ($\hat{p}=0.045-0.049$) to those of wheat, rye and barley, all belonging to another tribe Triticeae. This fact is in agreement with the fact that both tribes are included in the same subfamily, Pooideae. The present phylogenetic relationships among the four cereals of this subfamily are in agreement with those previously reported by Vedel *et al.* (1980),

Fig. 6. A phylogenetic tree of the family, Gramineae, that is based on conventional systematic data (cited from Tateoka 1957).

based on the results of cytoplasmic DNA variation. The chloroplast genome of foxtail millet belonging to the tribe Paniceae, sorghum in the tribe Andropogoneae, and job's tears and maize in Maydeae, all three tribes of which belong to the same subfamily, Panicoideae, had a $\hat{p}$ value of 0.021 to 0.043. The genetic distance observed between two species belonging to different tribes of the same subfamily was within the range of $\hat{p}=0.021-0.049$. On the other hand, the genetic distance observed between two species belonging to different subfamilies was within the range of $\hat{p}=0.053-0.144$. Thus, the genetic distance, $\hat{p}=0.05$, is regarded as a critical value for classifying two

species into different subfamilies.

The present results also revealed that among the four subfamilies, Eragrostoidae and Pooideae were the most closely related with each other, Panicoideae being next closest. Pharoideae was the most distant to all other subfamilies. These relationships are in complete agreement with their phylogenetic relationships determined by conventional systematic studies (cf. Figs. 5 and 6). In conclusion, the phylogenetic relationships among chloroplast genomes of the ten cereals revealed by the present restriction endonuclease analysis of their ctDNAs are in good agreement with those derived from the traditional systematic studies. This fact provides a sound basis for our future investigations on the phylogenetic origin of important crop species by the restriction endonuclease analysis of the organellar DNAs of the crop species and their wild relatives.

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