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Diversity and evolution of chloroplast DNA in *Triticum* and *Aegilops* as revealed by restriction fragment analysis *

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Summary. Restriction fragment analysis of chloroplast (cp) DNAs from 35 wheat (*Triticum*) and *Aegilops* species, including their 42 accessions, was carried out with the use of 13 restriction enzymes to clarify variation in their cpDNAs. Fourteen fragment size mutations (deletions/insertions) and 33 recognition site changes were detected among 209 restriction sites sampled. Based on these results, the 42 accessions of wheat-*Aegilops* could be classified into 16 chloroplast genome types. Most polyploids and their related diploids showed identical restriction fragment patterns, indicating the conservatism of the chloroplast genome during speciation, and maternal lineages of most polyploids were disclosed. This classification of cpDNAs was principally in agreement with that of the plasma types assigned according to phenotypes arising from nucleus-cytoplasm interactions. These mutations detected by restriction fragment analysis were mapped on the physical map of common wheat cpDNA, which was constructed with 13 restriction endonucleases. Length mutations were more frequently observed in some regions than in others: in a 16.0 kilo base pairs (kbp) of DNA region, including *rbcL* and *petA* genes, 6 of 14 length mutations were concentrated. This indicates that hot spot regions exist for deletions/insertions in chloroplast genome. On the other hand, 33 recognition site mutations seemed to be distributed equally throughout the genome, except in the inverted repeat region where only one recognition site change was observed. Base substitution rate (p) of cpDNA was similar to that of other plants, such as *Brassica*, pea and *Lycopersicon*, showing constant base substitution rates among related taxa and slow evolution of cpDNA compared with animal mitochondrial DNA. Phylogenetic relationships among *Triticum* and *Aegilops* species were discussed, based on the present data.

Key words: Evolution of cpDNA – Restriction fragment analysis – Wheat-*Aegilops* species – Phylogenetic relationships

Introduction

Since Correns (1909) first pointed out the existence of cytoplasmic genetic factors, namely plasmon, there has been an appreciation of the harmonic interaction of the nuclear genome with plasmon during the course of plant development and speciation (Grun 1976). In wheat, Kihara (1951) carried out pioneer work on nucleus-cytoplasm interactions using nuclear substitution lines of wheat. Fukasawa (1953) also introduced a new system of nucleus-cytoplasm interactions with *T. durum* nucleus and *Ae. ovata* cytoplasm. Later, Tsunewaki and coworkers (Tsunewaki 1980) systematically investigated the cytoplasmic effects on morphological, physiological and reproductive characters using alloplasmic lines of common wheats. Genetic diversity among the cytoplasms of almost all species in two related genera, *Triticum* and *Aegilops*, and the phenotypic effects of the nucleus-cytoplasm interaction were disclosed by those works. Consequently, cytoplasms of 33 species of *Triticum* and *Aegilops* were classified into 12 plasma types depending upon their biological effects on various characters of 12 different common wheat testers.

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Table 1. Plant sources used for the chloroplast DNA isolation

Code No.	Cytoplasm donor				Nucleus donor[b]	Plasma type[c]
	Species	Source[a]	Nuclear genome (n)			
			Ploidy	Constitution		
01	*T. monococcum*	K	2×	A	Mn	A
01 b	*T. urartu*	K	2×	A	Ur	–
02	*Ae. caudata*	K	2×	C	Cmp	C
03	*Ae, umbellulata*	K	2×	C^u	JF	C^u
04	*Ae. squarrosa*	K	2×	D	CS	D
05	*Ae. comosa*	K	2×	M	CS	M
06	*Ae. heldreichii*	P	2×	M	CS	M^h
07	*Ae. uniaristata*	M	2×	M^u	CS	M^u
13	*Ae. mutica*	M	2×	Mt	CS	Mt
14	*Ae. mutica*	P	2×	Mt	CS	Mt^2
08	*Ae. speltoides*	K	2×	S	CS	S
09	*Ae. aucheri*	P	2×	S	Splt	G
12	*Ae. bicornis*	M	2×	S^b	CS	S^b
10	*Ae. sharonensis*	K	2×	S^1	CS	S^1
18	*Ae. searsii*	K	2×	S^s	Ss	–
21	*T. dicoccoides*	K	4×	AB	CS	B
22	*T. dicoccum*	K	4×	AB	CS	B
23	*T. dicoccoides* var. nudigl.	K	4×	AG	Splt	G
24	*T. araraticum*	M	4×	AG	Splt	G
25	*T. timopheevi*	K	4×	AG	Splt	G
26	*T. triuncialis*	K	4×	CC^u	JF	C^u
38	*T. triuncialis*	P	4×	CC^u	Cmp	C
27	synthetic *triuncialis*	K	4×	CC^u	Cmp	C
28	*Ae. cylindrica*	K	4×	CD	CS	D
29	*Ae. biuncialis*	K	4×	C^uM^b	JF	C^u
37	*Ae. biuncialis*	P	4×	C^uM^b	JF	C^u
30	*Ae. columnaris*	K	4×	C^uM^c	JF	C^u
31	*Ae. ovata*	K	4×	C^uM^o	CS	M^o
32	*Ae. triaristata*	K	4×	C^uM^t	JF	C^u
33	*Ae. kotschyi*	K	4×	C^uS^v	CS	S^v
34	*Ae. variabilis*	K	4×	C^uS^v	CS	S^v
35	*Ae. crassa* 4×	M	4×	DM^{cr}	CS	D^2
36	*Ae. ventricosa*	K	4×	DM^v	CS	D
51	*T. zhukovskyi*	M	6×	AAG	Splt	G
52 a	*T. aestivum*	K	6×	ABD	CS	B
52 b	*T. aestivum*	K	6×	ABD	Srg	B
52 c	*T. spelta*	K	6×	ABD	Splt	B
53	*Ae. juvenalis*	K	6×	C^uDM^j	CS	D^2
54	*Ae. triaristata* 6×	K	6×	$C^uM^tM^{t2}$	JF	C^u
57	*Ae. triaristata* 6×	P	6×	$C^uM^tM^{t2}$	JF	C^u
55	*Ae. crassa* 6×	K	6×	DD^2M^{cr}	CS	D^2
56	*Ae. vavilovii*	K	6×	$DM^{cr}S^p$	CS	D^2

[a] K = Laboratory of Genetics, Kyoto University; M = S.S. Maan, North Dakota State University; P = I. Panayotov, Inst. Wheat Sunflower

[b] Nucleus donors are coded as follows: Mn = *T. monococcum;* Ur = *T. urartu;* Cmp = *T. compactum* cv No. 44; JF = *T. aestivum* cv Jones Fife; CS = *T. aestivum* cv Chinese Spring; Splt = *T. spelta* var. duhamelianum; Ss = *Ae. searsii;* Srg = *T. aestivum* cv Shirogane-komugi

[c] After Tsunewaki (1980); – = Plasma type not given

The major constituents of plasmon are chloroplasts (plastids) and mitochondria, both of which have their own self-replicating DNA. The basic structure of the chloroplast genome is well conserved among widely divergent plants (for review see Palmer 1985), and the chloroplast possesses a single circular molecule mostly within a range of 120–180 kilo base pairs (kbp) of DNA, in contrast to mitochondrial genomes, which consist of different molecular species generated by intramolecular recombination (Lonsdale et al. 1984), consequently yielding complicated restriction fragment patterns by restriction enzyme analysis (e.g., Pring and Levings 1978). The size of chloroplast (cp) DNA tends to be smaller in monocotyledonous plants than in dicotyledons. Compa-

rative studies on the evolution of cpDNA have been fewer in monocotyledonous plants than in dicots (Enomoto et al. 1985; Palmer 1985). As in most other plant taxa, the cpDNA of wheat is a 135 kbp circular molecule disrupted by 21 kbp inverted repeats. These facts encouraged us to perform restriction fragment analysis of cpDNA of wheat and its relatives (Ogihara and Tsunewaki 1982; Bowman et al. 1983; Tsunewaki and Ogihara 1983). Restriction fragment analysis of chloroplast DNAs has become a powerful new means for studying phylogenetic relationships among related plant species (Kung et al. 1982; Gordon et al. 1982; for review see Palmer 1985) as well as for determining the cytoplasm type of cybrids (e.g., O'Connell and Hanson 1985). We describe herein the classification of chloroplast genomes in almost all *Triticum* and *Aegilops* species and the mode of chloroplast DNA evolution of these species. These data provide a molecular basis for the diversity of cytoplasms seen among wheat and *Aegilops* species.

Materials and methods

Plant material

Thirty-six alloplasmic lines of common wheat (Tsunewaki 1980) and six euplasmic lines of *Triticum monococcum*, *T. urartu*, two *T. aestivum* cultivars, *T. spelta* and *Aegilops searsii*, which cover almost all species of the wheat-*Aegilops* complex as to their cytoplasms, were used for chloroplast DNA isolation. As shown in Table 1, they are grouped in order of the ploidy level of the cytoplasm donor. Since plasmon shows maternal inheritance in wheat, alloplasmic lines, so far as they are available, were used in the investigation because of their high seed productivity and uniform germination.

Chloroplast DNA isolation and restriction endonuclease analysis

Intact chloroplasts were isolated from leaves of the 42 lines, using the discontinuous Percoll (Pharmacia) gradient method (Ogihara and Tsunewaki 1982), from which chloroplast DNAs were extracted according to the method of Kolodner and Tewari (1975). CpDNAs were digested with 13 restriction endonucleases (*Bam*HI, *Bgl*II, *Hind*III, *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sca*I, *Sma*I, *Sst*I, *Xba*I and *Xho*I) as described by the manufacturer's instruction (Takara Shuzo) and fractionated by 0.8 or 1% agarose gel electrophoresis in TAE (40 m*M* Tris, 20 m*M* sodium acetate and 2 m*M* EDTA, pH 8.0).

Transfer of DNA to nitrocellulose filter and hybridization to ^{32}P-labelled probes

Restriction fragments were transferred to nitrocellulose filters (Schleicher and Schull BA85) as described by Maniatis et al. (1982). Recombinant plasmids were used as the hybridization probes. The DNA (0.2–0.5 μg) was labelled with [α^{32}P]dCTP by nick-translation (Rigby et al. 1977). The nitrocellulose filters were hybridized overnight in the hybridization buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 1 M NaCl, 100 μg/ml carrier DNA, 0.1% SDS, 10 × Denhardt's solution) at 65 °C for high stringency hybridization. After rinsing twice in 2 × SSC and 0.1% SDS solution at 65 °C for 15 min to remove unincorporated radioactive material, the nitrocellulose sheets were wrapped with Cling film and exposed to X-ray film with intensifying screen at − 70 °C for autoradiography.

In order to detect short DNA fragments, filters were hybridized overnight at 60 °C and washed at 60 °C using the same buffer as described above.

Cloning wheat cpDNA fragments and construction of physical map

PstI-digested wheat cpDNA (*T. aestivum* cv Asakaze komugi) was cloned into the *Pst*I site of pBR322 using *Escherichia coli* strain HB101. Resultant recombinant plasmid DNAs were prepared (Birnboim and Doly 1979) and their inserts checked by size fractionation using agarose gel electrophoresis. Ten independent clones containing P3–P12 fragments of wheat cpDNA (Bowman et al. 1981) were obtained. Further recombinant plasmids, i.e., pTacB(3–11), pTacB(11–12), pTacB2, pTacB5, pTacB(10–18) and pTacB(8–9), were kindly supplied by Bowman and Dyer (1986) to complete the cpDNA bank. Recognition sites of ten restriction enzymes were determined by double and/or partial digestion of these plasmid DNAs or Southern hybridization of whole cpDNA and/or the cloned plasmid DNAs. A physical map of wheat cpDNA obtained with three

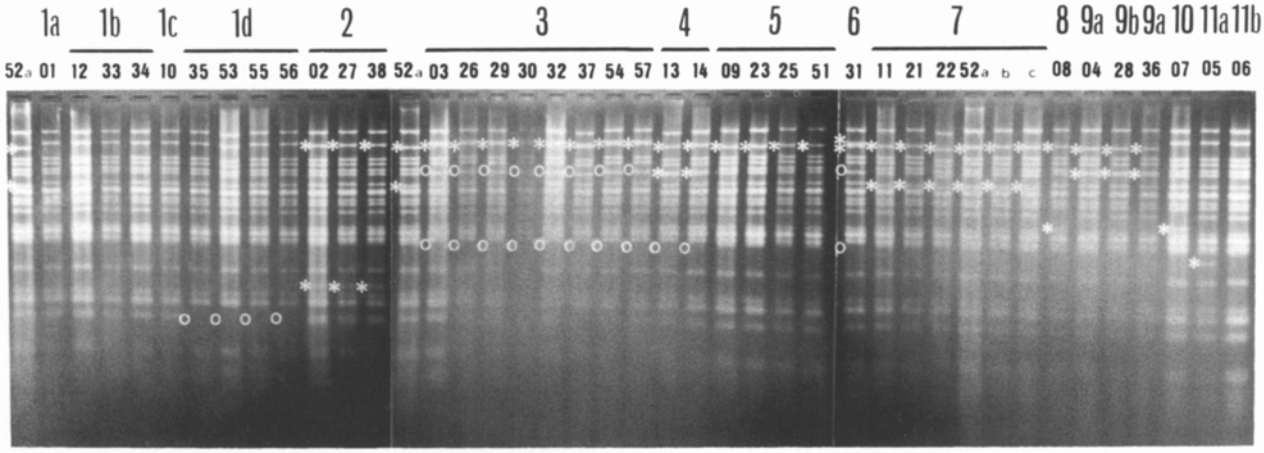

Fig. 1. *Bam*HI restriction fragment patterns of cpDNAs from 41 *Triticum* and *Aegilops* cytoplasms. * and o: Fragments changed and missing, respectively, compared with the ones in chloroplast genome type 1 b (see Tabel 4 for classification)

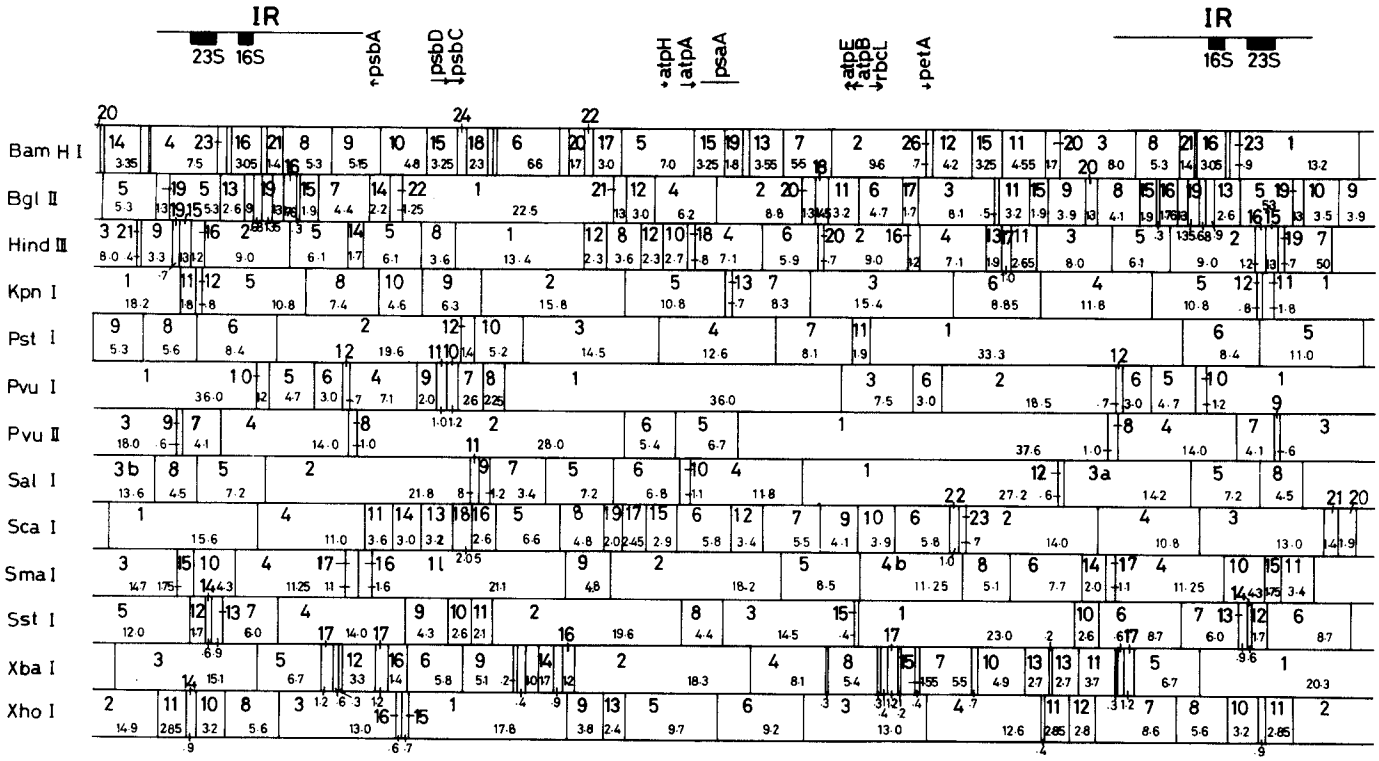

Fig. 2. Physical map of wheat chloroplast DNA showing recognition sites of 13 restriction enzymes and sizes in kbp of the individual fragments

restriction enzymes (*Bam*HI, *Pst*I, *Sal*I) was used as reference (Bowman et al. 1983).

Results

With the use of 13 restriction endonucleases, restriction fragment patterns of cpDNAs were obtained from the 42 lines of *Triticum-Aegilops* species and compared with each other. As an example of the results, the patterns of *Bam*HI fragments after 0.8% agarose gel electrophoresis are presented in Fig. 1. Of three possible chloroplast DNA mutations, i.e., deletions/insertions (fragment size mutations), restriction site changes (point mutations) and inversions/translocations (fragment rearrangements), the first two types are detectable by the present method and are dealt with in this paper.

Construction of physical map of T. aestivum cpDNA with 13 restriction enzymes

Based on the first physical map of wheat cpDNA (*Triticum aestivum* cv. Madler) constructed by Bowman et al. (1981, 1983), who used the three restriction enzymes *Bam*HI, *Pst*I and *Sal*I, further mapping of the restriction sites of ten additional restriction endonucleases was worked out (Fig. 2). The recognition sites of the 13 en-

Table 2. Fragment size mutations revealed in chloroplast DNAs of 42 *Triticum* and *Aegilops* species (or accessions), detected by use of 13 restriction endonucleases

Mutation[a]	Location[b]	Size (kbp)	Carrier cytoplasms (Code No.)
Insertion			
i1	B4-H9[b]	0.1	12
i2	B10-Sc14	0.1	01, 01 b
i3	B6-Xb9	0.1	all except 21, 22, 52 a, b, c
i4	B13-Sc12	0.1	10
i5	B7-Bl20	0.2	all except 21, 22, 52 a, b, c
i6	B7-Bl20	0.1	01, 01 b
i7	B2-Bl6	0.9	01, 01 b, 02, 03, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 29, 30, 32, 33, 34, 35, 37, 38, 53, 54, 55, 56, 57
Deletion			
d1	S7 & S9	0.2	05, 06, 07
d2	B5-H8	0.2	04, 28, 36
d3	B2-Sc6	0.3	02, 27, 38
d4	B2-P11	0.1	05
d5	B2-Sc10	0.2	04, 28, 36
d6	Bl2-Sc6	0.4	08
d7	Bl2-Sc6	0.35	07

[a] Insertions and deletions given here are all with reference to *T. aestivum* chloroplast DNA

[b] Fragment designations are given in Fig. 2. B = *Bam*HI, Bl = *Bgl*II, H = *Hin*dIII, P = *Pst*I, S = *Sal*I, Sc = *Sca*I, and Xb = *Xba*I

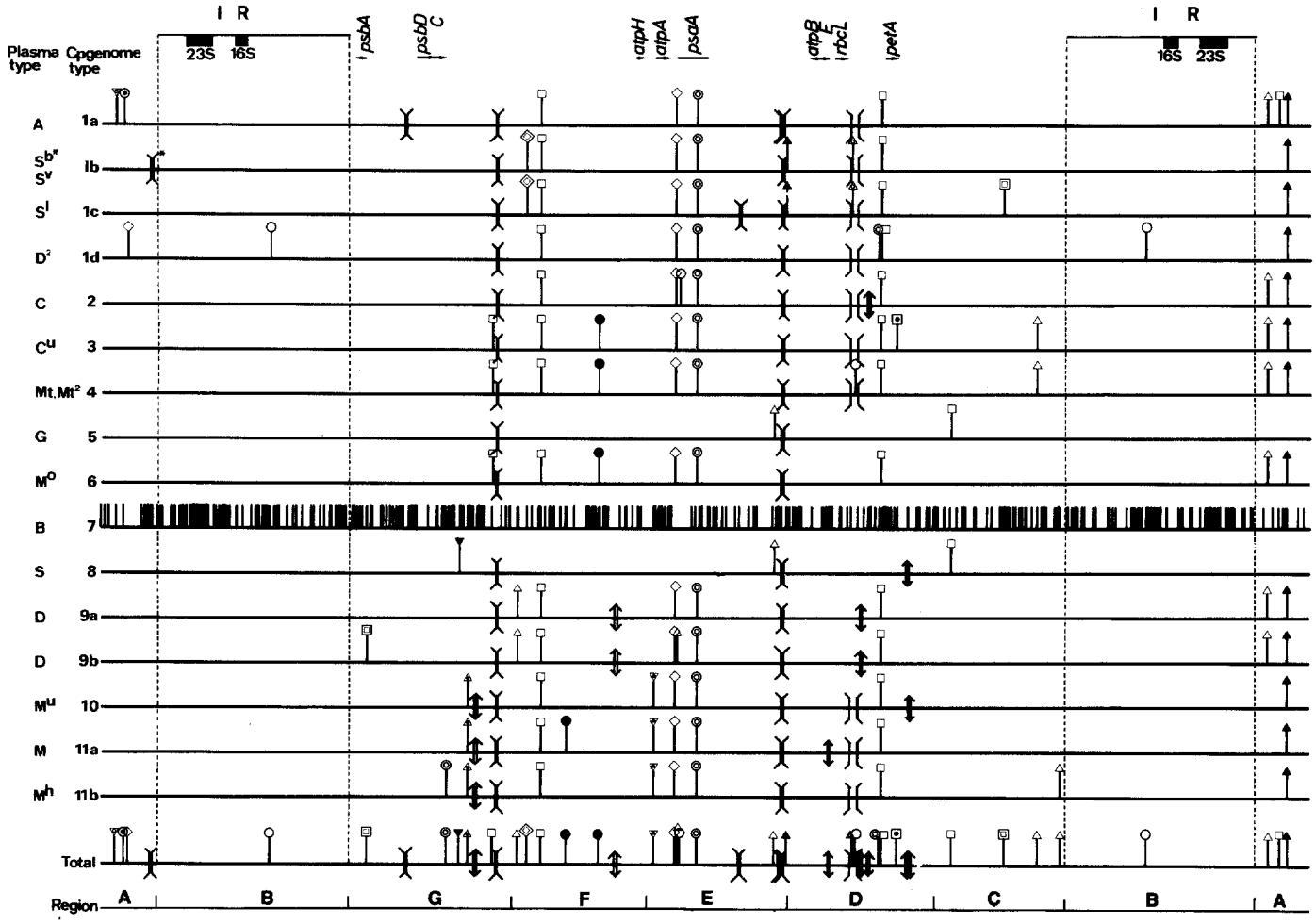

Fig. 3. Locations of the length and restriction site mutations observed among 16 chloroplast genome types on the physical map of common wheat chloroplast DNA (genome type 7). ⇕ and)(indicate a deletion and insertion, respectively, as compared with *T. aestivum*. The symbols for restriction site changes are as follows: ○, ● = *Bam*HI, △, ▲ = *Bgl*II, ◇, ◆ = *Hin*dIII, ▽ ▼ = *Pst*I, ⍋, ⍋ = *Pvu*I, ▽, ▼ = *Pvu*II, ⊓, ▀ = *Sma*I, ◈, ◈ = *Sst*I, ◎, ◉ *Xba*I, ⊡, ⊡ = *Xho*I site. The first and second symbols represent a site gain and loss, respectively for the respective endonuclease. *: Only the chloroplast genome of *Ae. bicornis* harbors this insertion

zymes were distributed almost equally over the entire wheat chloroplast genome, although there were some regions where restriction enzymes rarely recognized their sites of action (Fig. 3).

Deletions and insertions

Fourteen different deletions and insertions were detected in the present DNAs (Table 2). With reference to the cpDNA map of common wheat (Fig. 2), 13 were localized in the large single copy region, only 1 was in the small single copy region and none were in the inverted repeat regions (Fig. 3). A 9.6 kbp fragment of the *Bam*HI digests, termed the B2 fragment, varied in size from 10.5–9.45 kbp among lines of the wheat-*Aegilops* complex (Fig. 4). In total, four sizes of mutations were found in this fragment, compared to the *T. aestivum* cpDNA (B2 = 9.6 kbp). These were a 0.9 kbp insertion found in 27 cytoplasms (i7 in Table 2), double size mutations, namely, 0.9 kbp insertion (i7) + 0.3 kbp deletion (d3) in three cytoplasms, and a 0.2 kbp deletion (d5) in three other cytoplasms. These mutations resulted in B2l (10.5 kbp), B2m (10.2 kbp) and B2s fragment (9.4 kbp), respectively. A fragment similar in size (10.0 kbp) was found in ten cytoplasms. This fragment was produced by loss of a *Bam*HI site between B5 and B17 fragment (Table 3), and was not related to B2 fragments as proved by Southern hybridization. Three other deletions (d4, d6 and d7) were closely located to these insertions and deletions. These results dramatically indi-

Table 3. Restriction site changes detected for 10 out of the 13 restriction endonucleases used

Enzyme	No. fragments scored	Designation of mutation	Changed fragments (kbp)		Carrier cytoplasm
			Lost	Gained	
*Bam*HI	37	Bm1	9.6	6.8+3.7	13, 14
		Bm2	3.25	2.0+1.3	02, 27, 38
		Bm3	1.4	0.75+0.65	35, 53, 55, 56
		Bm4	7.3+3.0	10.0	03, 13, 14, 26, 29, 30, 31, 32, 37, 57
		Bm5	1.7+1.0	2.7	05
*Bgl*II	33	Bl1	22.5	15.2+7.3	04, 28, 36
		Bl2	6.2	5.1+1.1	28
		Bl3	4.1	4.0+0.1	06
		Bl4	3.9	2.9+0.9	03, 13, 14, 26, 29, 30, 32, 37, 54, 57
		Bl5	3.5	1.85+1.65	01, 01 b, 02, 03, 04, 13, 14, 26, 27, 28, 29, 30, 31, 32, 36, 37, 38, 54, 57
		Bl6	1.3	0.7+0.6	08, 09, 23, 25, 51
		Bl7	3.9+3.5	7.4	01, 01 b, 02, 03, 04, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 53, 54, 55, 56, 57
		Bl8	1.45+1.3	2.75	10, 12, 18, 33, 34
*Hind*III	28	Hn1	7.1	6.2+0.9	01, 01 b, 02, 03, 04, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 53, 54, 55, 56, 57
		Hn2	8.0	6.8+1.1	35, 53, 55, 56
*Pst*I	12	Ps1	5.2+1.4	6.6	08
*Pvu*I	15	PvI1	7.5	4.5+3.0	10, 12, 18, 33, 34
		PvI2	2.6+2.25	4.2	05, 06, 07
*Pvu*II	9	PvII1	18.0	10.6+7.5	01, 01 b
		PvII2	6.7+5.4	12.1	05, 06, 07
*Sma*I	15	Sm1	21.1	20.8+0.8	01, 01 b, 02, 03, 04, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 53, 54, 55, 56, 57
		Sm2	20.8	14.7+5.8	03, 13, 14, 26, 29, 30, 31, 32, 37, 54, 57
		Sm3	14.7	13.1+1.6	01, 01 b
		Sm4	11.25	7.3+5.0	01, 01b, 02, 03, 04, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 53, 54, 55, 56, 57
		Sm5	5.1	2.9+2.2	08, 09, 23, 24, 25, 51
*Sst*I	17	Ss1	19.6	14.5+5.1	10, 12, 18, 33, 34
*Xba*I	34	Xb1	18.3	17.5+0.8	01, 01 b, 02, 03, 04, 05, 06, 07, 10, 12, 13, 14, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 53, 54, 55, 56, 57
		Xb2	5.8	5.7+0.1	06
		Xb3	1.55	1.4+0.1	35, 53, 55, 56
		Xb4	20.3+15.1	35.5	01, 01 b
*Xho*I	19	Xh1	13.0	10.5+2.5	28
		Xh2	12.6	12.2+0.4	10
		Xh3	14.0+12.6	27.0	03, 26, 29, 30, 32, 37, 54, 57

cate that this region, covered by B2, B26 and B12 fragments (region D in Fig. 3), is a hot spot for size mutations. Three insertions (i2, i5 and i6) were found adjacent to this region (Fig. 3). Two insertions (i2 and i3) and one deletion (d1) were clustered in a region (Region G in Fig. 3) close to an inverted repeat, showing this region to be a second deletion/insertion hot spot. We could not always detect a size mutation with all 13 restriction enzymes. For example, size alterations in the B2 region were observed with 9 of the 13 enzymes used; that is, the other 4 enzymes, *Pst*I, *Pvu*II, *Sal*I and *Sst*I, did not reveal any size alterations in this region located in the P1 (33.3 kbp), PvII1 (37.6 kbp), S1 (27.2 kbp) and Ss1 fragment (23.0 kbp), respectively (Fig. 2).

Restriction site changes

The other type of mutation found was a gain or loss of restriction sites. In total, 33 restriction site mutations (Table 3) were detected. No size alterations were ob-

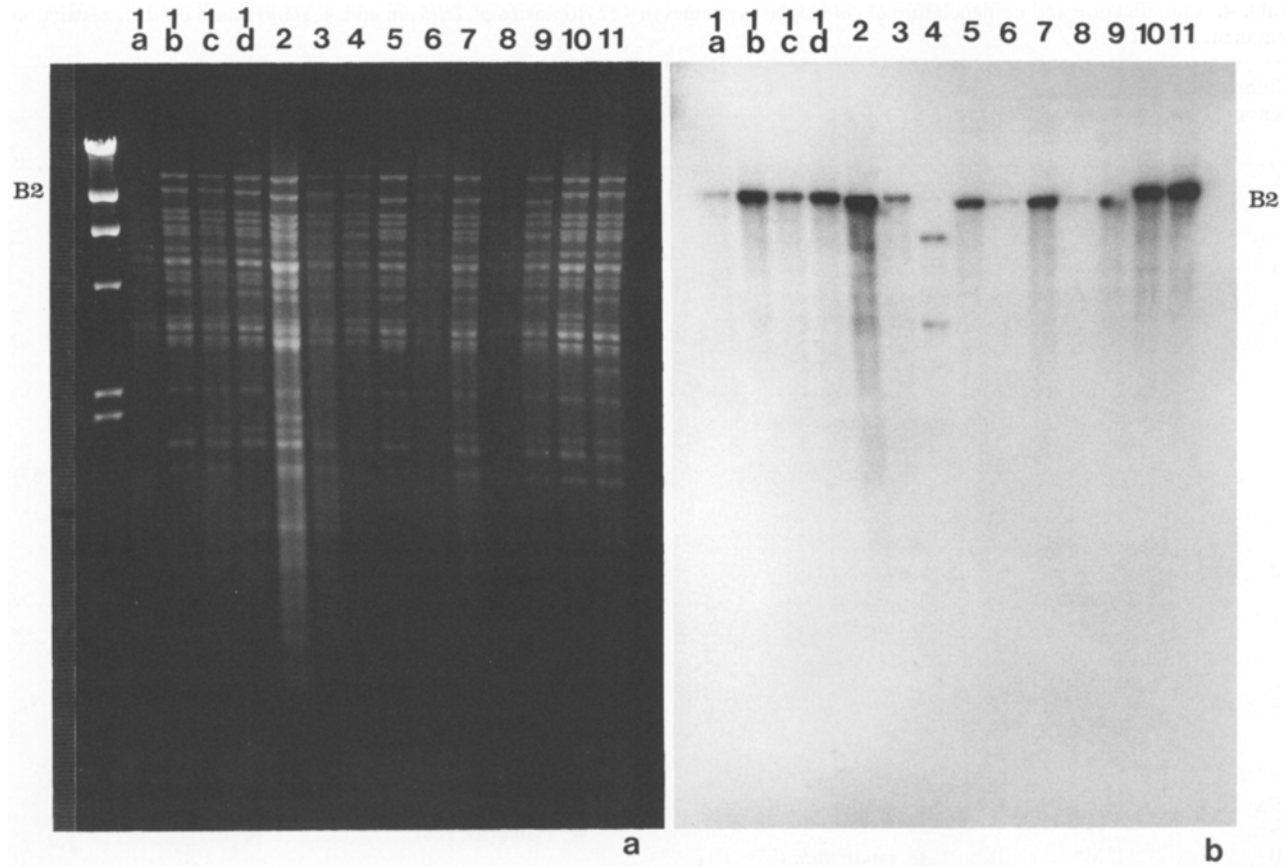

Fig. 4a and b. *Bam*HI restriction fragment patterns of 14 chloroplast genome types (**a**) and their Southern hybridization with the fragment B2 of common wheat as a probe (**b**)

served in these mutated fragments. In each mutation of this category, two fragments replaced by a new fragment in the case of a site loss and two new fragments substituted for a missing fragment in the case of a site gain were all adjacent to each other on the physical map (Fig. 2). All fragments involved in the mutations were confirmed by Southern hybridization using the clones of the corresponding wheat fragments as probes. The positions of all the mutated restriction sites thus deduced are indicated in Fig. 3. We cannot, however, rule out the possibility that some of these mutations are caused by small deletions/insertions involving the restriction sites under consideration.

Discussion

Classification of chloroplast genome in Triticum-Aegilops complex and origin of the cytoplasms of individual polyploid species

Chloroplast DNAs of some cytoplasms produced identical restriction fragment patterns with each other (Fig. 1, Tables 2 and 3). For example, cpDNAs of *Ae. comosa* (05), *Ae. heldreichii* (06) and *Ae. uniaristata* (07) all possessed the same 0.2 kbp deletion in the fragment containing *Sal*I site between S7 and S9, as compared with all other DNAs. CpDNAs of *Ae. caudata* (02), synthetic *Ae. triuncialis* (27) and *Ae. triuncialis* (38) had a new *Bam*HI recognition site in a B15 fragment (3.25 kbp), producing two new fragments. From the results given in Tables 2 and 3, cpDNA's from 42 cytoplasms of *Triticum* and *Aegilops* were classified into 16 chloroplast genome types (Table 4). Referring to the previous nomenclature of 11 chloroplast genomes from 38 cytoplasms of *Triticum* and *Aegilops* (Ogihara and Tsunewaki 1982), we propose a supplemented chloroplast genome nomenclature shown in Table 4.

Evolutionary conservatism of the chloroplast genome (Palmer et al. 1985) is strongly supported by the comparison of cpDNAs between diploids and their related polyploid species. In most cases, we were able to find diploid species whose chloroplast genome was identical to that of individual polyploid species. For example, cpDNAs of *Ae. aucheri* and an *Ae. speltoides* accession were identical to those of the Timopheevi wheat group. CpDNAs of *T. urartu* and *Ae. seasii* were identical and/or mostly sim-

Table 4. Classification and nomenclature of chloroplast genomes in 42 cytoplasms of *Triticum* and *Aegilops* based on their restriction fragment patterns

Chloroplast genome type	Plasma[a] type	Carrier species (Code number)		
		Diploid	Tetraploid	Hexaploid
1a	A	*T. monococcum* (01) *T. urartu* (01b)	–	–
1b	S^b	*Ae. bicornis* (12)	–	–
1b	S^v	*Ae. searsii* (18)	*Ae. kotschyi* (33) *Ae. variabilis* (34)	–
1c	S^1	*Ae. sharonensis* (10)	–	–
1d	D^2	–	*Ae. crassa* 4× (35)	*Ae. juvenalis* (53) *Ae. crassa* 6× (55) *Ae. vavilovii* (56)
2	C	*Ae. caudata* (02)	*Ae. triuncialis* (38) Syn. *triuncialis* (27)	–
3	C^u	*Ae. umbellulata* (03)	*Ae. triuncialis* (26) *Ae. biuncialis* (29, 37) *Ae. columnaris* (30) *Ae. triaristata* 4× (32)	*Ae. triaristata* 6× (54, 57)
4	Mt	*Ae. mutica* (13)	–	–
	Mt^2	*Ae. mutica* (14)	–	–
5	G	*Ae. aucheri* (09)	*T. dic'des nudigl.* (23) *T. araraticum* (24) *T. timopheevi* (25)	*T. zhukovskyi* (51)
6	M^o	–	*Ae. ovata* (31)	–
7	B	–	*T. dic'des spont.* (21) *T. dicoccum* (22)	*T. aestivum* (52a, b) *T. spelta* (52c)
8	S	*Ae. speltoides* (08)	–	–
9a	D	*Ae. squarrosa* (04)	*Ae. ventricosa* (36)	–
9b	D	–	*Ae. cylindrica* (28)	–
10	M^u	*Ae. uniaristata* (07)	–	–
11a	M	*Ae. comosa* (05)	–	–
11b	M^h	*Ae. heldreichii* (06)	–	–

[a] After Tsunewaki (1980)
[b] The plasma type of *Ae. searsii* has not been biologically determined

ilar to those of *T. monococcum* and *Ae. bicornis*, respectively, greatly differing from those of Emmer-common wheat groups. *Ae. ventricosa* had an identical cpDNA to that of *Ae. squarrosa*. These facts suggest that most chloroplast genomes differentiated at the diploid level and have not changed appreciably after polyploidization. There are, however, a few interesting exceptions. The first one is intraspecific variability in *Ae. triuncialis*. The cpDNA of one *Ae. triuncialis* accession (code no. 26) shows identical restriction fragment patterns to those of *Ae. umbellulata*, whereas that of the second accession (no. 38) has patterns identical to that of *Ae. caudata*. These facts indicate that *Ae. triuncialis* was produced diphyletically by the reciprocal crosses between *Ae. caudata* and *Ae. umbellulata*. In addition, Murai and Tsunewaki (1986) found a third cpDNA type in *Ae. triuncialis* which differs from the cpDNA types of both *Ae. caudata* and *Ae. umbellulata*. As a second exception, we could not find, so far, any diploid species having cpDNAs identical to those of Emmer-common wheats (chloroplast genome type 7), *Ae. ovata* (type 6), *Ae. cylindrica* (type 9b) and *Ae. crassa* complex (type 1d), including the third type of *Ae. triuncialis*. Efforts to search for the diploid carriers of their cpDNAs among wheat-*Aegilops* collections should be made.

Based on the present results (Table 4, Fig. 5 and later section of "Discussion"), the origin of four chloroplast genomes found only in some polyploid species will be considered here. Chloroplast genome 1d of *Ae. crassa* (and two other species) has no close relatives, differing by at least six mutations from all other types. So, no assumptions can be made as to its origin. Genome type 6 of *Ae. ovata* closely resembles genome type 3 of *Ae. umbellulata* and type 4 of *Ae. mutica*, and, therefore, either of those diploids or an unknown species related to them seems to be the cytoplasm donor of *Ae. ovata*. Chloroplast genome 7 of Emmer and common wheats most resembles type 5 and 8 genomes of *Ae. speltoides*, including *Ae. aucheri*; and chloroplast genomes of all other diploids, including *Ae. bicornis*, *Ae. sharonensis* and *Ae. searsii*, differ greatly from it. Therefore, it is definitely concluded that the cytoplasm of Emmer wheat is derived

from some form of *Ae. speltoides*. Based on an earlier study of cpDNA of alloplasmic common wheat (Tsunewaki and Ogihara 1983), we reported that an *Ae. longissima* cytoplasm has cpDNA identical to that of Emmer cytoplasm. Later works (unpublished) suggested this "alloplasmic" wheat was mistagged during its production by repeated backcrosses, having common wheat cytoplasm at present. The chloroplast genome 9b of *Ae. cylindrica* closely resembles the genome type 9a of *Ae. squarrosa*, and this diploid is assumed to have been the cytoplasm donor of *Ae. cylindrica*.

Nature of cpDNA mutations in Triticum and Aegilops

A characteristic feature of chloroplast DNA evolution in the *Triticum* and *Aegilops* complex is the relatively high incidence of length mutations as in *Brassica* (Palmer et al. 1983). We detected 14 different length mutations (Table 2), in contrast to 33 restriction site changes among 209 recognition sites investigated (Table 3). Gordon et al. (1982) and Bowman et al. (1983) reported a higher ratio of length mutations to site changes in cpDNA evolution in *Oenothera* and *Triticum-Aegilops*, respectively. However, their level of sensitivity for detection of base changes was much less than ours because they studied only 36 and 48 sites, respectively. Palmer and Zamir (1982) reported the rare occurrence of length mutations in *Lycopersicon* cpDNA evolution; of 40 fragment changes found among 484 bands, 39 were of restriction site changes. Palmer et al. (1985) reported a high incidence of length mutations and rearrangements in *Pisum* cpDNAs; they detected 17 length mutations as opposed to 11 restriction site mutations among 165 restriction sites sufficiently covering the entire chloroplast genome. Evidently, the relative proportion of the length mutation over the site mutation in the wheat-*Aegilops* complex is intermediate between those of *Lycopersicon* and *Pisum*. At the bottom (on the line "total") of Fig. 3, all mutations detected among the 16 chloroplast genome types are depicted. Also, 209 restriction sites identified in *T. aestivum* cpDNA are drawn on the line of chloroplast genome type 7. The length mutations occurred frequently in specific regions, such as D and G, whereas the site mutations are distributed almost evenly throughout the entire chloroplast genome except in the inverted repeat regions, where only one mutation was detected. To confirm these facts, mutations detected in seven regions of cpDNA are scored (Table 5). As stated in "Results", three regions, E, G and particularly D, are considered as hot spots for length mutations. Such hot spots are reported in *Nicotiana* (Tassopulu and Kung 1984), *Oenothera* (Gordon et al. 1982), and *Pisum* (Palmer et al. 1985). In the former two genera, hot spots are located in the helical position of the large single copy region close to inverted repeat around the *psbA* gene. In the latter, on the other hand, variable regions are positioned close to the *petA* and *rbcL-psbA* genes. Inversions are known between cpDNAs of dicotyledons and monocotyledons (Palmer 1985). Major breakage points are assumed to be close to *psbA* and *petA* genes, when spinach cpDNA is taken as a standard. At the endpoint of this inversion, short repeated sequences homologous to *att*-lambda are found in wheat (Howe 1985). Day and Ellis (1984) proposed that cpDNA deletions produced during plant regeneration from wheat pollen grains occurred by an intramolecular recombination between homologous segments of cpDNA, as is known to occur in mitochondrial DNA of maize (Londsdale et al. 1984). In fact, Bowman and Dyer (1986) recently found 12 kinds of small dispersed repeats in wheat cpDNA, of which their No. 5 seems to correspond to a segment deleted in our d5. Furthermore, Palmer et al. (1987) pointed out the role of repeats in the unusual evolution of the chloroplast genome of the geranium. Although the positions of these chloroplast genome alterations were not exactly the same as those of our length mutations, these hot spot regions seem to contain some specific sequences leading to occasional intramolecular recombinations.

Restriction site differences and conservatism of chloroplast genome

The number of restriction site mutations was scored using the data presented in Table 3, and the base substitution rate p ($100\times$) per base pair in the all recognition sites investigated was calculated after Brown et al. (1979) (Table 6). The overall average of p, 0.66, of wheat-*Aegilops* cpDNA is similar in order to those of two other taxa, i.e., 0.38 in *Pisum* (Palmer et al. 1985) and 0.87 in *Brassica* (Palmer et al. 1983). When compared with animal mitochondrial DNA, a strikingly low base substitution rate of chloroplast DNA is disclosed, because the rates of *Drosophila* and primate mitochondrial DNAs are 4.86 and 25, respectively (DeSalle et al. 1986, Brown et al. 1979). It should be emphasized that the value of *Drosophila* is of intraspecies variability in *D. sylvestris*. We may conclude that the rate of base substitution in the chloroplast genome is low in contrast to the high incidence of genome structural alterations detected. DNA sequence data of cpDNA around the rbcL *gene* (Zurawski et al. 1984) supports this suggestion.

Phylogenetic relationship among 16 chloroplast genome types of Triticum and Aegilops

Using the unweighted pair-group clustering (UPG) method (Sneath and Sokal 1973) on the data presented in Tables 2 and 3, we have constructed a phylogenetic tree for the 16 chloroplast genomes (Fig. 5). Recurrent occurrence of two mutations, i.e., i7 and Bl5, is assumed

Table 5. No. of mutations located in seven regions of cpDNA found among 42 *Triticum* and *Aegilops* cytoplasms

Region[a]	Size (kbp)	No. insertions/ deletions	No. restrict. sites observed	No. mutated sites	Mutations per restrict. site
A: SSCR	12.8	1	20	6	0.30
B: IR	21 (×2)	0	61	1	0.02
C: LSCRI (Sm14-Sc23)	16.5	0	38	4	0.11
D: LSCRII (Sc23-S1)	16.0	6	35	7	0.20
E: LSCRIII (S4-H10)	15.5	3	28	5	0.18
F: LSCRIV (H12-P3)	15.7	1	32	5	0.16
G: LSCRV (P10-Sm16)	16.5	3	45	5	0.11
Total	135.0	14	259	33	0.13

[a] SSCR, IR, and LSCR = Small single copy region, inverted repeats and large single copy region, respectively

Table 6. Restriction site differences observed and base substitution rates estimated among 16 wheat-*Aegilops* chloroplast genome types. The no. of restriction site differences between every pair of chloroplast DNAs is shown in the upper right half of this table. The matrix given in the lower left half of the table is the base substitution rate per 100 base pairs in the recognition sites which were calculated after Brown et al. (1979). In total, 1,254 bp were counted for estimating this rate

Cp genome types	1a	1b	1c	1d	2	3	4	5	6	7	8	9a	9b	10	11a	11b	Average
1a		7	8	7	4	7	7	11	5	9	12	4	6	6	7	8	7.2
1b	0.70		1	6	5	8	8	10	6	8	11	5	7	5	6	7	6.7
1c	0.78	0.39		7	6	9	9	11	7	9	12	6	8	6	7	8	7.6
1d	0.70	0.62	0.70		5	8	8	10	6	8	11	5	7	5	6	7	7.1
2	0.47	0.55	0.62	0.54		5	5	9	3	7	10	2	4	4	5	6	5.3
3	0.70	0.78	0.86	0.78	0.55		2	12	2	10	13	5	7	7	8	9	7.5
4	0.70	0.78	0.86	0.78	0.54	0.31		12	2	10	13	5	7	7	8	9	7.5
5	0.86	0.79	0.86	0.78	0.71	0.94	0.94		10	2	1	9	11	9	10	11	9.2
6	0.55	0.63	0.70	0.62	0.39	0.31	0.31	0.79		8	10	3	5	5	6	7	5.7
7	0.71	0.63	0.71	0.63	0.55	0.79	0.79	0.16	0.63		3	7	9	7	8	9	7.6
8	0.94	0.87	0.94	0.86	0.79	1.03	1.02	0.08	0.87	0.24		10	12	10	11	12	10.1
9a	0.47	0.55	0.62	0.54	0.31	0.55	0.54	0.71	0.39	0.55	0.79		2	4	5	6	5.2
9b	0.62	0.70	0.78	0.70	0.47	0.70	0.70	0.86	0.54	0.71	0.94	0.31		6	7	8	7.1
10	0.63	0.55	0.63	0.55	0.47	0.71	0.71	0.71	0.55	0.56	0.79	0.47	0.63		1	2	5.6
11a	0.71	0.63	0.71	0.63	0.55	0.79	0.79	0.79	0.63	0.64	0.88	0.55	0.71	0.24		3	6.5
11b	0.78	0.71	0.78	0.70	0.63	0.86	0.86	0.87	0.71	0.71	0.95	0.63	0.78	0.32	0.40		7.5
Average	0.69	0.66	0.73	0.68	0.54	0.71	0.71	0.72	0.58	0.60	0.80	0.53	0.68	0.57	0.64	0.71	0.66

(Fig. 5). To critically confirm this, the nucleotide sequencing of the regions containing these mutations is necessary.

Among the 16 chloroplast genome types, the following five primary clusters are formed: (1) 1b (*Ae. bicornis* and *Ae. searsii* group) and 1c (*Ae. sharonensis*); (2) 10 (*Ae. uniaristata*), 11a (*Ae. comosa*) and 11b (*Ae. heldreichii*); (3) 5 (*T. timopheevi* group), 7 (*T. aestivum* group) and 8 (*Ae. speltoides*); (4) 9a (*Ae. squarrosa* group) and 9b (*Ae. cylindrica*); and (5) 3 (*Ae. umbellulata* group), 4 (*Ae. mutica* group) and 6 (*Ae. ovata*). Chloroplast genome types 1d (*Ae. crassa* group) and 2 (*Ae. caudata* group) form a secondary cluster with two primary clusters, (4) and (5). This secondary cluster is supposed to be in the center of *Triticum* and *Aegilops* plastom diversity because it includes the most primitive species, *Ae. mutica*. It is noteworthy that the primary cluster consisting of chloroplast genome types 5, 7 and 8 shows the greatest separation from the supposed ancestral group. This plastom relationship shows some differences from the plasmon relationship based on the phenotypic effects of all plasmons on various morphological and physiological characters of alloplasmic wheats (Tsunewaki 1980). Although some part of this difference may be due to mitochondrial genome diversity, the present phylogenetic tree of the chloroplast genome (Fig. 5) might more properly reflect the relationship among different plasmons than their dendrogram depicted based on phenotypic effects.

A discrepancy was found between the traditional classification and chloroplast genome relationships between Sitopsis species. *Ae. aucheri* (a form of *Ae. spel-*

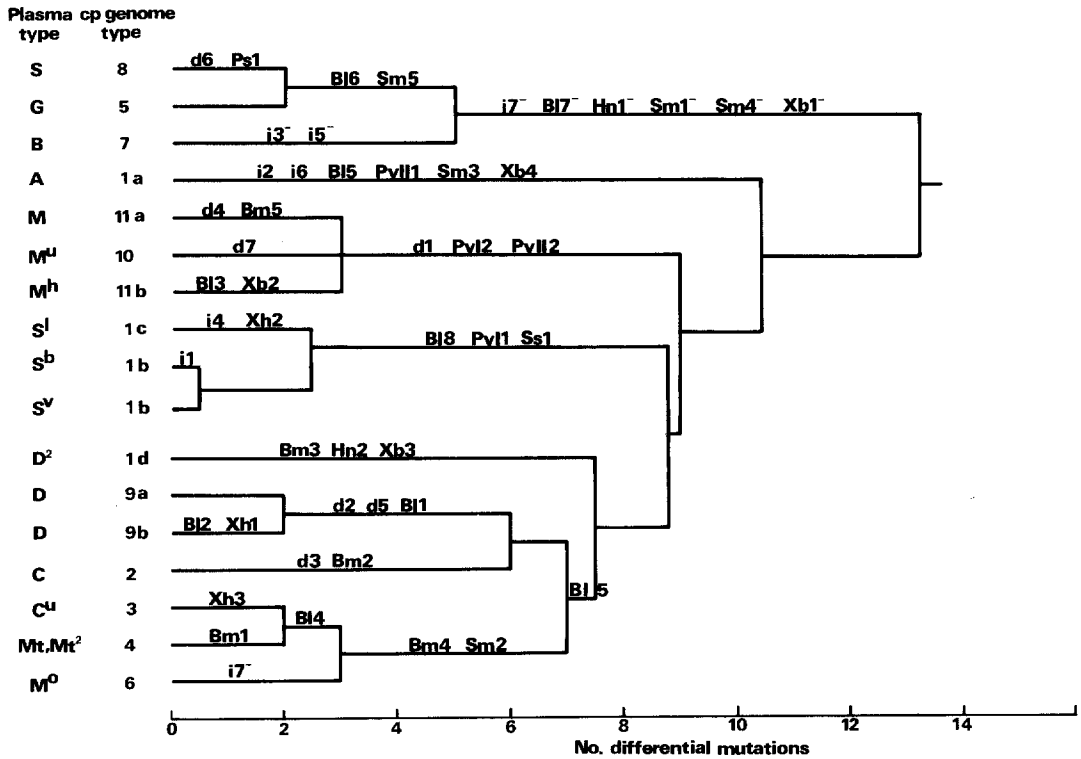

Fig. 5. A dendrogram showing phylogenetic relationships among the 16 chloroplast genome types constructed by the UPG method using total numbers of restriction fragments changes observed between every pair of genomes. Mutational events including deletion/insertion (Table 2) and recognition site changes (Table 3) that differentiate individual branches of the tree are indicated. The minus sign (−) indicates the lack of an indicated mutation in the corresponding branch (namely, presence of the indicated mutation in all other branches)

toides), Ae. speltoides, Ae. bicornis, Ae. searsii, Ae. longissima and *Ae. sharonensis* are morphologically similar, having nuclear genomes more or less related to each other (Kihara 1954; Kihara and Tanaka 1970). Their chloroplast genomes, however, are separated into two distinct groups, namely, one consisting of *Ae. aucheri* (type 5) and *Ae. speltoides* (type 5 and 8) of subsection Truncata, and the other comprising *Ae. bicornis* (type 1 b), *Ae. sharonensis* (type 1 c) and *Ae. searsii* (type 1 b) of the subsection Emarginata (*Ae. longissima* was not studied). Systematic and genetic relationships between these two subsections must be reinvestigated.

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