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Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm

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Summary. Restriction endonuclease analysis revealed interspecific and intraspecific variation between the chloroplast DNAs and therefore between the cytoplasms of 14 selected species of *Triticum* and *Aegilops*. Eleven distinct chloroplast DNA types were detected, the differences between them residing in the varied combination of a relatively few DNA alterations.

The variation was simple enough for chloroplast DNA analysis to be used as a basis for the identification and classification of the *Triticum* and *Aegilops* cytoplasms. There was good agreement with the classification based on analysis of the phenotypic effects of the cytoplasm when combined with the *T. aestivum* nucleus in nuclear-cytoplasmic hybrids (Tsunewaki et al. 1976). There was however no correlation between specific chloroplast DNA alterations and any of the phenotypic effects known to be associated with specific cytoplasms.

Although the diploid species examined included all those which have been suggested as possible donors of the cytoplasm and the B genome to *T. aestivum*, none of the chosen accessions belonged to the same cytoplasmic class as *T. aestivum* itself, except that of the tetraploid *T. dicoccoides*. Therefore, none of the diploid accessions analysed was the B genome donor. The analyses did however support several other suggestions which have been made concerning wheat ancestry. Scoring the different chloroplast DNA types according to the rarity of their banding patterns indicated that four of the eleven cytoplasms are of relatively recent origin.

The DNA alterations most easily detectable by the limited comparison of the eleven *Triticum/Aegilops* chloroplast DNA types using only 4 endonucleases were insertions and deletions. These ranged between approximately 50 bp and 1,200 bp in size and most of them were clustered in 2 segments of the large single-copy region of the genome. Only two examples of the loss of restriction endonuclease sites through possible point mutations were observed. No variation was detected in the inverted repeat regions. Several of the deletions and insertions map close to known chloroplast protein genes, and there is also an indication that the more variable regions of the chloroplast genome may contain sequences which have allowed DNA recombination and rearrangement to occur.

Key words: *Triticum* – *Aegilops* – Chloroplast DNA – Cytoplasmic inheritance – Wheat evolution – The B genome donor – Restriction mapping – Insertions – Deletions

Introduction

The harmony of interaction between genes encoded in nuclear DNA and those encoded in the DNA of cytoplasmic organelles has a fundamental influence on the growth and development of higher plants. The probable cause of this influence is the chimaeric nature of the organelles themselves; the subunits of several of their holoproteins are now known to be coded for by organellar and nuclear genes (Bedbrook et al. 1976; Bedbrook et al. 1980; Ellis 1981; Parthier 1982).

The importance of the interaction between these organellar and nuclear genes is readily observed in nuclear-cyto-

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plasmic (N–C) hybrid plants, when a nonharmonious interaction can cause phenotypic effects such as leaf variegation, male and/or female sterility, severe growth inhibition, enhanced vegetative growth or killing of cold-sensitive hybrid plants in winter (Tsunewaki et al. 1976). Perhaps the best known and most economically important example of such an effect is the male sterility conferred by the 'Texas' cytoplasm in *Zea mays* (Duvick 1965; Miller and Koeppe 1971).

To understand more fully the part played in this interaction by the cytoplasmic genes, detailed studies have been made by constructing N–C hybrids in which the hexaploid wheat nucleus has been combined, by many backcross generations, with the cytoplasm of several of its close relatives from the genera *Triticum* and *Aegilops* (Tsunewaki et al. 1980). This approach has revealed a considerable genetic diversity among the *Triticum* and *Aegilops* cytoplasms and has indicated that certain of the phenotypic effects are associated with specific cytoplasmic types. The differences and similarities of the phenotypic effects have been used as a basis to classify the cytoplasms of *Triticum* and *Aegilops* and to study their phylogenetic relationships.

In the study reported here we have surveyed the chloroplast DNAs (ctDNAs) of selected *Triticum* and *Aegilops* species by restriction endonuclease analysis. One of our aims was to evaluate ctDNA analysis as a basis for cytoplasmic classification using the "phenotypic effect" classification of Tsunewaki et al. (1980) as a framework. It was also of interest to see whether any of the phenotypic effects associated with specific cytoplasmic types could be related to a particular ctDNA organisation.

An extensive study of cytoplasmic inheritance in *Triticum* and *Aegilops* species has shown that the ancestral diploid donor of the cytoplasm to the hexaploid wheat *T. aestivum* was also the donor of the B genome (Tsunewaki et al. 1980). We therefore included in this survey examples of those diploid species which have been put forward as possible B genome donors.

The main aim of this study was to use the comparative ctDNA analyses to examine the pattern of heterogeneity in *Triticum/Aegilops* chloroplast genomes. To date, some of the most valuable contributions to our understanding of the role of chloroplast genes in higher plants have come from studies of the relatively few higher plant chloroplast genome mutants which have been described (e.g. Hagemann and Börner 1978; Kutzelnigg and Stubbe 1974; Simpson et al. 1977). Such chloroplast genome mutants are however extremely difficult to induce and often lethal. Therefore, as an alternative, we hoped to identify different *Triticum* and *Aegilops* wild type ctDNAs which would be useful for future study of the detailed organisation, and the in vivo regulation and interactions of higher plant chloroplast genes.

Materials and methods

Plant material

The species, populations and varieties of *Triticum* and *Aegilops* used in this survey were as follows:

Hexaploid breadwheat *T. aestivum* (haploid genome – ABD) varieties; 'Mardler', 'Cappelle-Desprez', 'Highbury' and 'Chinese Spring'.

Tetraploid wheats; *T. dicoccoides* (– AB) and *T. araraticum* and *T. timopheevi* (– AG).

Diploid wheats: *T. boeoticum*, *T. monococcum* and *T. urartu* (–A).

Diploid goatgrasses; *Ae. longissima* (– S^1), *Ae. bicornis* (– S^b), *Ae. sharonensis* (– S^1), *Ae. searsii* (– S^s) and *Ae. speltoides* (– S) PBI accessions H^2, M, K, S, T and V.

Depending on the availability of seed, one hundred to two hundred seedlings of each type were grown in seed trays as previously described (Bowman and Dyer 1982) to a height of about 10 cm, and then placed in the dark or in dim light for 24 h to destarch the leaves. The seedlings were harvested, frozen in liquid N_2 and stored at – 80 °C. To collect sufficient material from some of the less vigorous species, the seedlings were allowed to grow up several times to a 10 cm-height and harvested successively. A freeze-dried powder was made from the pooled leaves as previously described (Bowman and Dyer 1982).

Preparation and restriction endonuclease analysis of ctDNA

Chloroplasts were isolated from 0.5 g samples of the freeze-dried powder using a nonaqueous procedure (Bowman and Dyer 1982) with the modification that powdered glass was added to the freeze-dried powder during grinding to increase the efficiency of homogenisation and therefore the yield of chloroplasts. The isolation depends on isopycnic banding of the chloroplasts in a hexane/carbon tetrachloride step-gradient. The purity of the chloroplasts and hence of the ctDNA, is determined by the specific gravity of the "overlay" gradient step above which the chloroplasts are banded. The chloroplast preparations from most of the species examined were sufficiently pure when banded using an overlay step of specific gravity 1.32. However, with material from *T. dicoccoides*, *T. boeoticum*, *Ae. longissima*, *Ae. sharonensis* and *Ae. squarrosa*, it was necessary to reduce the specific gravity of the overlay step to 1.30 to obtain sufficiently pure chloroplasts.

Total nucleic acid was extracted from the chloroplast pellets, aliquots of 20–30 µg (containing 0.2–0.5 µg ctDNA) were incubated with the restriction endonucleases PstI, and SalGI, BamHI or EcoRI in the presence of pancreatic A ribonuclease and the digestion products were fractionated by electrophoresis on horizontal 0.85% agarose slab gels as previously described (Bowman and Dyer 1982). DNA fragment sizes were also determined as previously described (Bowman et al. 1981).

Transfer of DNA fragments to nitrocellulose and hybridisation to ^{32}P-labelled probes

Chloroplast DNA fragments, fractionated in 3 mm-thick 0.85% agarose gels, were transferred to Schleicher and Schüll BA85 nitrocellulose sheets after partial hydrolysis as described by Wahl et al. (1979). The procedure was slightly modified in that the gels were incubated only once with 0.25 M HCl, and 0.5 M NaOH alone was used as the neutralizing solution. The nitrocellulose sheets were stored at 4° after washing in 10×SSC and baking for 2 h at 80°. DNA (0.1 to 0.2 µg) to be

used for probes was labelled with ^{32}P by nick translation (Rigby et al. 1977). Prehybridisation and hybridisation were carried out with constant shaking in 50% formamide medium at 42 °C as described by Wahl et al. (1979). After hybridisation, the nitrocellulose sheets were rinsed twice with 2×SSC at room temperature, and unhybridised material was removed by two 30 min washes in 2×SSC, 0.1% SDS at 65 °C. The sheets were dried and exposed to X-ray film at −80 °C using tungsten intensifying screens.

Results

Comparative restriction endonuclease analysis

Interspecific and intraspecific variation in ctDNA. Agarose gel electrophoresis of fragments produced by terminal digestion of ctDNA with restriction endonuclease BamHI, PstI, SalGI, or EcoRI is illustrated in Figs. 1–5. The gel shown in each figure is a summary of ctDNA digests representative of each of the *Triticum* and *Aegilops* species included in this survey. Where bands are marked with a white dot, their mobility has been analysed on several gels to establish that the DNA fragment constituting the band is different from the equivalent fragment in digests of *T. aestivum* ctDNA. The mobility of DNA bands in agarose gels is influenced not only by the size of the DNA fragment but also by the amount of DNA loaded and by gel edge-effects. Both these were therefore taken into account when estimating fragment sizes. Determinations were analysed statistically (Table 2), but care was also taken to confirm differences in fragment size by comparing DNA samples containing approximately the same quantity of DNA, run in adjacent tracks, away from the edge of the gel. To show the reproducibility of band variation, some examples of duplicate digests are shown in Figs. 1 and 2 for *T. aestivum, T. urartu, Ae. longissima, Ae. searsii* and *Ae. speltoides.* Digests of *T. aestivum* ctDNA were electrophoresed in the outer tracks of each gel. When digests of ctDNA from the four different varieties of *T. aestivum* ('Mardler', 'Cappelle-Desprez', 'Highbury' and 'Chinese Spring') were

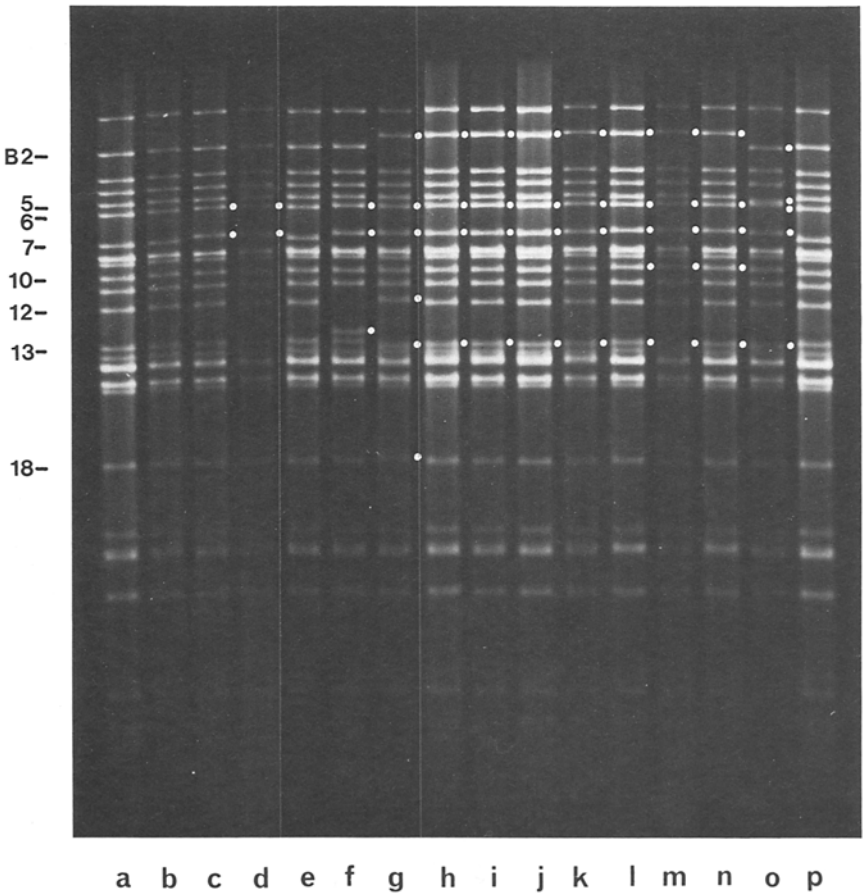

Fig. 1. BamHI digests of ctDNA from several species *Triticum* and *Aegilops,* fractionated by agarose gel electrophoresis. (*a*) (*e*) (*p*) *T. aestivum,* (*b*) *T. dicoccoides,* (*c*) *T. timopheevi,* (*d*) *T. araraticum,* (*f*) *Ae. speltoides* M,(*g*) *Ae. speltoides* S, (*h*) *Ae. searsii,* (*i*) *Ae. longissima,* (*j*) *Ae. bicornis,* (*k*) *Ae. sharonensis,* (*l*) *T. urartu,* (*m*) *T. monococcum,* (*n*) *T. boeoticum,* (*o*) *Ae. squarrosa.* Bands marked on the right with a white dot are variants of the *T. aestivum* ctDNA bands numbered on the left

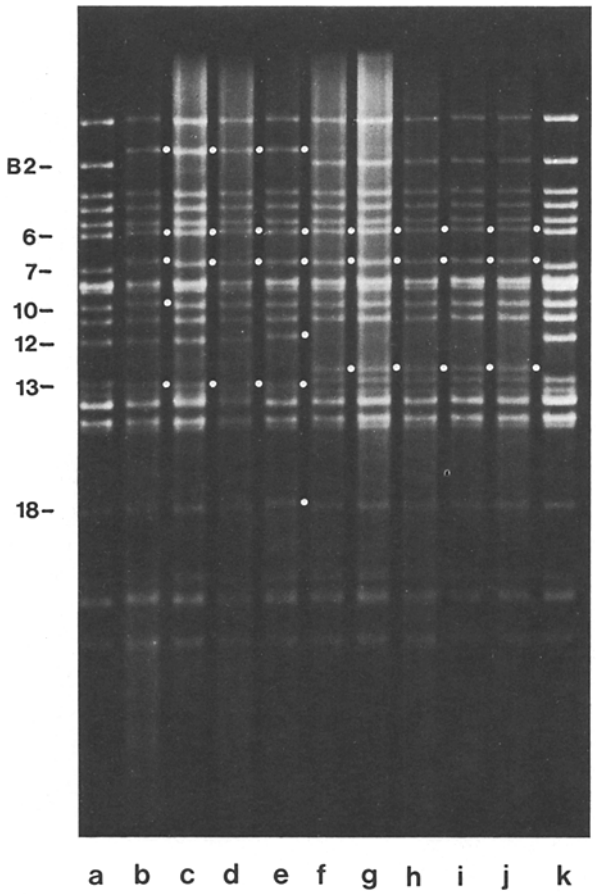

Fig. 2. BamHI digests of ctDNA from several species of *Triticum* and *Aegilops* and several accessions of *Ae. speltoides*, fractionated by agarose gel electrophoresis. (*a*) (*k*) *T. aestivum*, (*b*) *T. urartu*, (*c*) *Ae. longissima*, (*d*) *Ae. searsii*, (*e*)–(*j*) *Ae. speltoides* accessions (*e*) S (*f*) V (*g*) T (*h*) K (*i*) M and (*j*) H_2. Bands marked on the right with a white dot are variants of the *T. aestivum* ctDNA bands numbered on the left

compared, no differences were detected. 'Mardler' is the variety represented in Figs. 1–5.

BamHI fragments. For purposes of comparison, the restriction endonuclease which produced the most informative distribution of the *Triticum/Aegilops* ctDNA bands was BamHI. Figures 1 and 2 show that using this endonuclease alone, interspecific variation in the ctDNA banding pattern is readily detectable. Nevertheless, the overall pattern is also so similar, that one can often assume that a change in band mobility reflects a difference in the size of a ctDNA fragment cleaved from the same region of the genome. When a restriction endonuclease is used to compare ctDNAs from less closely related species, or if an enzyme is used which gives a more variable banding pattern, it is usually necessary to establish the relationship between variable bands using cross hybridisation techniques (as shown in Fig. 7). Of the 23 distinct bands generated by cleavage of ctDNA with BamHI, 8 showed variation between the 14 species represented in Fig. 1 (B2, B5, B6, B7, B10, B12, B13 and B18). Four of these bands have 2 size variants (B5, B6, B10 and B18), and four have 3 size variants (B2, B7, B12 and B13). The sizes of the variant fragments are detailed in Table 1. The sizes previously determined for two of the *T. aestivum* ctDNA Bam fragments were found to be inaccurate (Bowman et al. 1981): B6 is 6.6 kbp and not 6.3 kbp, and B19 is 1.82 kbp and not 2.08 kbp.

Cleavage of the ctDNA with BamHI also revealed intraspecific variation in the 6 populations of *Ae. speltoides* that were analysed (Fig. 2). Two ctDNA patterns were observed. The common pattern is shown by 5 of the populations: *Ae. speltoides* V, T, K, M, and H_2 (Fig. 2 tracks (f)(g)(h)(i) and (j) respectively) whereas a unique fragment pattern was shown by *Ae. speltoides* S (Fig. 2 track (e)). *Ae. speltoides* M is used to represent the first group in Figs. 3–5. On the basis of this variation in ctDNA fragment patterns produced by digestion with BamHI alone, the ctDNAs of the species analysed in this survey could be classified into 7 types. There is evidently a relationship between these and the cytoplasmic types assigned to the species by Tsunewaki et al. (1980):

(i) *T. aestivum* and *T. dicoccoides* (cytoplasmic type S),
(ii) *T. timopheevi* and *T. araraticum* (G),
(iii) 5 populations of *Ae. speltoides* (G or S),
(iv) *Ae. speltoides* S (G or S),
(v) *Ae. searsii*, *Ae. longissima*, *Ae. bicornis* and *Ae. sharonensis* (S),
(vi) *T. urartu*, *T. monococcum* and *T. boeoticum* (A), and
(vii) *Ae. squarrosa* (D).

PstI and SalGI fragments. The endonucleases PstI and SalGI were used in the survey because, although they generate fewer ctDNA fragments and are therefore potentially less discriminating than EcoRI or BamHI, the recognition sites for both these enzymes have been located on the *T. aestivum* chloroplast genome (Bowman et al. 1981). Banding patterns of ctDNAs digested with PstI are shown in Fig. 3. Six of the 12 ctDNA fragments were found to vary in size (P7, P8, P9, P10, P11 and P12) and two to vary in number (P5 and P8).

Figure 4 shows a similar summary gel comparing SalGI ctDNA digests. SalGI proved to be the least discriminating of the 4 endonucleases. Only two of the Sal fragments (S6 and S7) were measurably variable in size and two (S5 and S9) were variable in number. Details of the variant Sal and Pst fragments are also given in Table 1.

Table 1. Variations in fragment size detected by restriction endonuclease analysis of ctDNA from several species of *Triticum* and *Aegilops*

Species	ctDNA type	ctDNA derivation score[b]	Fragment identity[a]																						
			B2	B5	B6	B7	B10	B12	B13	B18	P5	P7	P8	P9	P10	P11	P12	S5	S6	S7	S9	E2a	E3	E8/E4a		E13
T.aestivum	B	592	9.6	7.0	6.6	5.5	4.8	4.18	3.55	2.3	11.0	8.1	5.6	5.3	5.2	1.9	1.4	7.2	6.8	6.1	1.2	−1	4.4	2.8	−1	2.18
T. dicoccoides	B																									
T. timopheevi	G	641			6.7	5.76						8.4			5.3					6.2			4.5			
T. araraticum	G	641			6.7	5.76						8.4			5.3					6.2			4.5			
Ae. speltoides M	S	633			6.7	5.76		3.65				8.4			5.3					6.2			4.5			
Ae. speltoides S	S^2	613	10.3		6.7	5.76		4.25	3.42	2.35		8.4			5.3	1.95	1.45			6.2			4.5	−1	3.4	
Ae. sharonensis	S^1	648	10.3		6.7	5.76			3.42			8.4			5.3					6.2			4.5	−1	3.4	
Ae. searsii	S^1	648	10.3		6.7	5.76			3.42			8.4			5.3					6.2			4.5	−1	3.4	
Ae. longissima	$S^{1\,2}$	643	10.3		6.7	5.76			3.5		+1	8.4	−1	−1	5.3					6.2			4.5	−1	3.4	
Ae. bicornis	S^b	634	10.3		6.7	5.76			3.5			8.4	5.7		5.3					6.2			4.5	−1	3.4	
T. boeoticum	A	609	10.3		6.7	5.85	4.9		3.5			8.4		5.35	4.9			+1		−1	−1		4.15	−1	3.4	
T. monococcum	A^1	634	10.3		6.7	5.85	4.9		3.5			8.4			5.3					6.2			4.5	2.65	3.4	
T. urartu	A^2	643	10.3		6.7	5.85	4.9		3.5			8.4			5.3					6.2			4.5	−1	3.4	
Ae. squarrosa	D	620	9.1	6.8	6.7	5.76			3.5			8.4			5.3				6.6	6.2		4.55	4.5	−1	−1	−1

[a] B, P, S and E: fragments produced by cleavage of ctDNA with BamHI, PstI, SalGI and EcoRI respectively. Fragments are numbered in order of decreasing size and sizes are means given in kbp. SD were ± 0.029 for the B2 fragments and < ± 0.015 for the other fragments

[b] To estimate the derivation of the different ctDNA types each was scored as follows: a fragment common to all 11 types scored 11, a unique fragment scored 1, etc. For a total ctDNA score, the scores of each fragment detected in that type were summed

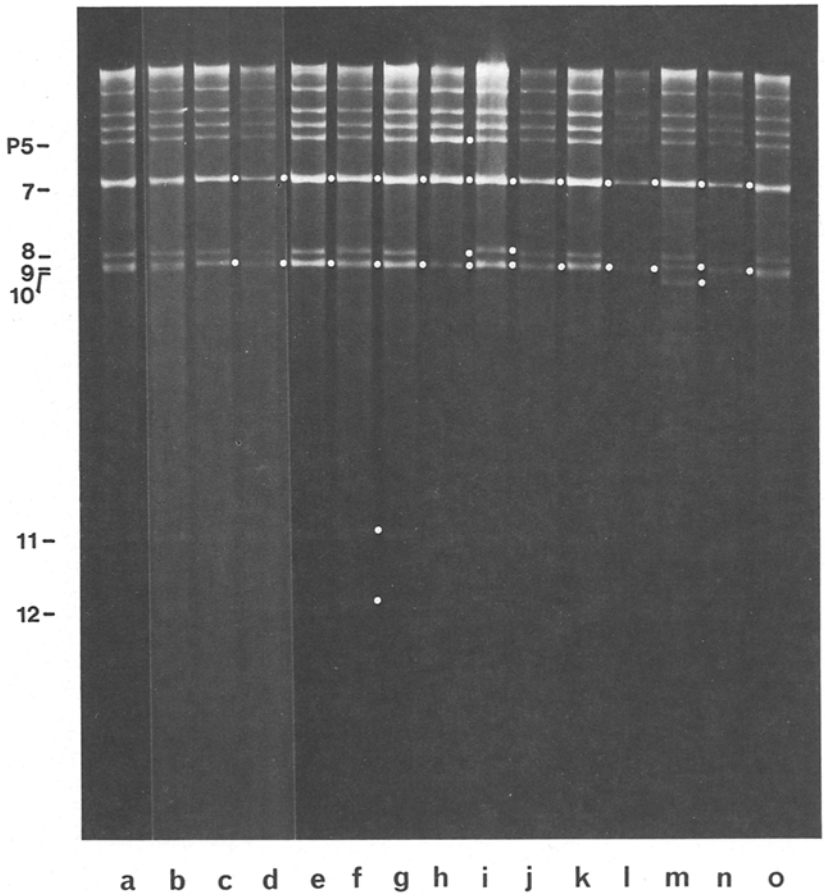

Fig. 3. PstI digests of ctDNA from several species of *Triticum* and *Aegilops*, fractionated by agarose gel electrophoresis. (*a*) (*o*) *T. aestivum*, (*b*) *T. dicoccoides*, (*c*) *T. timopheevi*, (*d*) *T. araraticum*, (*e*) *Ae. speltoides* M, (*f*) *Ae. speltoides* S, (*g*) *Ae. searsii*, (*h*) *Ae. longissima*, (*i*) *Ae. bicornis*, (*j*) *Ae. sharonensis*, (*k*) *T. urartu*, (*l*) *T. monococcum*, (*m*) *T. boeoticum*, (*n*) *Ae. squarrosa*. Bands marked on the right with a white dot are variants of the *T. aestivum* ctDNA bands numbered on the left

EcoRI fragments. Digests of the ctDNAs with EcoRI are compared in Fig. 5. Although we have little information on the precise mapping of the EcoRI recognition sites, EcoRI was used to allow a direct comparison of the data reported in this paper with other analyses of *Triticum* and *Aegilops* ctDNAs (e.g. Vedel et al. 1978; Vedel et al. 1981). However, because the resolution of the smaller EcoRI fragments (E16 and smaller) was inadequate on 0.85 % agarose, most deductions were made comparing only the larger fragments (E1 to E15 inclusive) even though some differences were also evident among the smaller fragments. The sizes of most of the EcoRI fragments 1 to 15 have not previously been determined for wheat ctDNA. They are, in kbp: E1, 13.0; E2, 7.0; E3, 4.4; E4, 3.7; E5, 3.1; E6, 2.85; E7, 2.8; E8, 2.68; E9, 2.46; E10, 2.35; E11, 2.30; E12, 2.25; E13, 2.18; E14, 2.08; E15, 1.90.

Of the 15 bands compared, four were obviously variable: E3, E4a, E8 and E13. A new band (E2a) appeared above E3 in the *Ae. squarrosa* ctDNA digest (Fig. 5 track m). Again, these variants are detailed in Table 1.

Although each individual restriction endonuclease differed in its ability to discriminate between the ctDNAs of the different cytoplasmic types, the combined data can be used to classify the ctDNA types, and hence the cytoplasms, as shown in Tables 1 and 2. Table 1 details the variation in ctDNA banding patterns revealed between all the species examined by analysis with all 4 enzymes. In total, sixty fragments were analysed and of these, the 23 listed in Table 1 were measurably variable in length. This degree of variation was sufficient to sort the cytoplasms into 11 different groups: B, G, S, S^1, S^2, S^{12}, S^b, A, A^1, A^2 and D. Each group is described by the symbol for the nuclear genome of the diploid species from which the cytoplasm originates (Tsunewaki et al. 1976). At this level of resolution and with the cytoplasmic types represented in this survey, 7 of the cytoplasms could be defined unequivocally, those of *Ae. squarrosa*, *T. boeoti-*

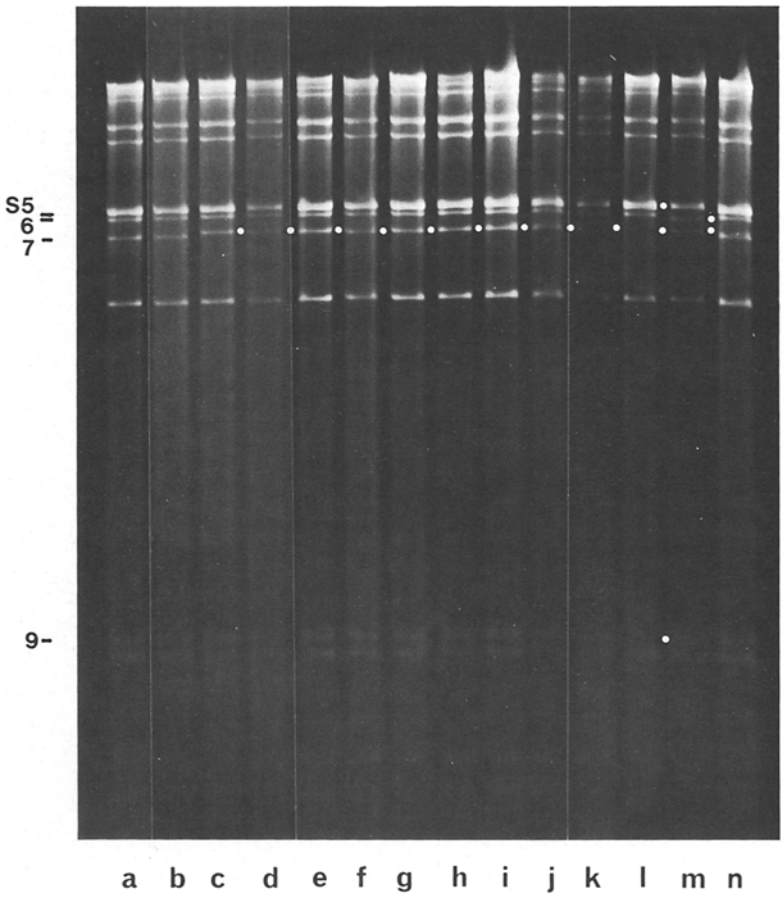

Fig. 4. SalGI digests of ctDNA from several species of *Triticum* and *Aegilops*, fractionated by agarose gel electrophoresis. (*a*) (*n*) *T. aestivum*, (*b*) *T. dicoccoides*, (*c*) *T. timopheevi*, (*d*) *T. araraticum*, (*e*) *Ae. speltoides* M, (*f*) *Ae. speltoides* S, (*g*) *Ae. searsii*, (*h*) *Ae. longissima*, (*i*) *Ae. bicornis*, (*j*) *Ae. sharonensis*, (*k*) *T. monococcum*, (*l*) *T. boeoticum*, (*m*) *Ae. squarrosa*. Bands marked on the right with a white dot are variants of the *T. aestivum* ctDNA bands numbered on the left

cum, *T. monococcum*, *T. urartu*, *Ae. bicornis*, *Ae. longissima* and *Ae. speltoides* S. *Ae. searsii* ctDNA was indistinguishable from that of *Ae. sharonensis*, so they are both included in the *sharonensis* cytoplasmic group S^1. Similarly, *T. araraticum* and *T. timopheevi* are placed together in group G, and *T. dicoccoides* and *T. aestivum* are assigned to group B. Therefore, among the 14 species studies, it was possible to detect 11 variants of the *Triticum/Aegilops* chloroplast genome.

To estimate the derivation of the different ctDNA types, each was scored as follows: a fragment common to all 11 types scored 11, a unique fragment score 1, etc. For a total ctDNA "derivation" score, the scores of each fragment detected in that type were summed. The total scores are shown in the third column of Table 1. The ctDNA types showing the lower scores contain more of the unique and rare bands and are therefore assumed to be of relatively recent origin. This is a fairly rough guide, since the sample is small and there are several ways of arriving at the same score. It does suggest, however, that of the eleven ctDNA types described in this paper, seven are of a similar antiquity, scoring 633–648, while four are of more recent origin scoring 620 or less: type D, type S^2, type A and type B.

Location of the variation of the Triticum/Aegilops ct genome

The serial order of the Sal and Pst fragments in the ctDNA of *T. aestivum* is shown in Fig. 6 (Bowman et al. 1981). Although ctDNA is a circular molecule, the map is drawn in linear form with the S2/P2 limit of the inverted repeat at the ends. The positions of the larger Bam fragments and two of the EcoRI fragments are also shown. These were determined by analysis of the products of Bam/Pst and Bam/Sal double digestions of cloned DNA of the individual Pst and Sal fragments. BamHI and EcoRI digests were also hybridised with ^{32}P-labelled probes made from isolated Sal fragments or from cloned Pst fragments (as in Fig. 7). The 2.68 kbp fragment E8, containing the ribulose bisphosphate carboxylase (RuBPCase) large subunit (LS) gene

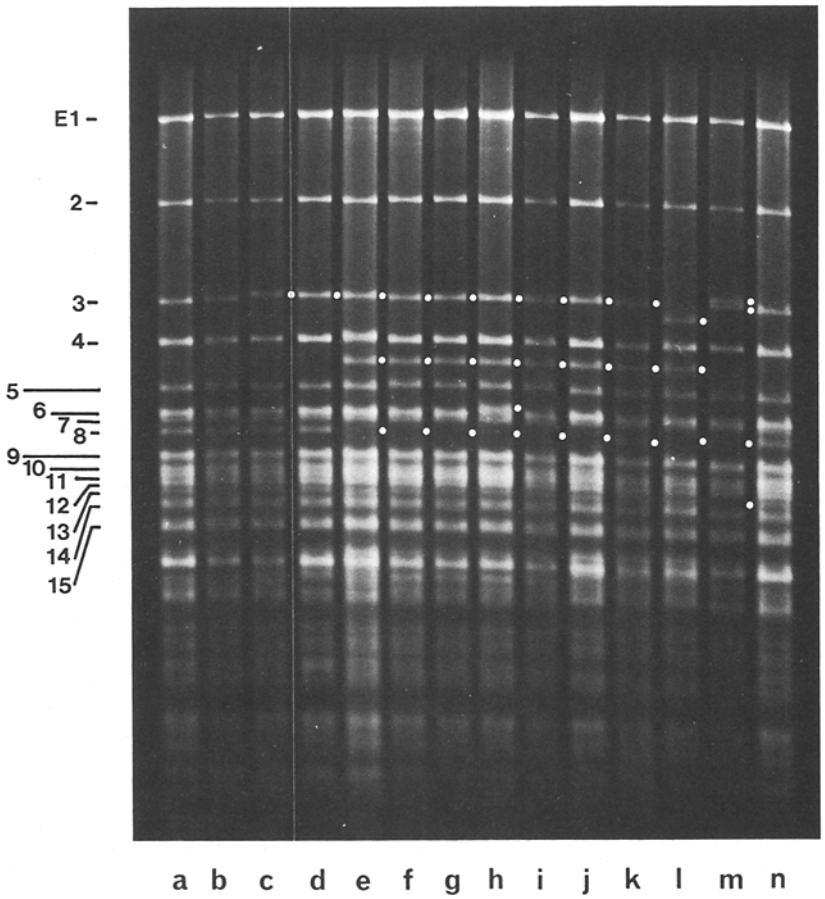

Fig. 5. EcoRI digests of ctDNA from several species of *Triticum* and *Aegilops*. (*a*) (*n*) *T. aestivum*, (*b*) *T. dicoccoides*, (*c*) *T. timopheevi*, (*d*) *Ae. speltoides* M, (*e*) *Ae. speltoides* S, (*f*) *Ae. searsii*, (*g*) *Ae. longissima*, (*h*) *Ae. bicornis*, (*i*) *Ae. sharonensis*, (*j*) *T. urartu*, (*k*) *T. monococcum*, (*l*) *T. boeoticum*, (*m*) *Ae. squarrosa*. *T. aestivum* ctDNA bands 1 to 15 are numbered on the left. Bands marked on the right with a white dot are variants of the equivalent *T. aestivum* ctDNA band

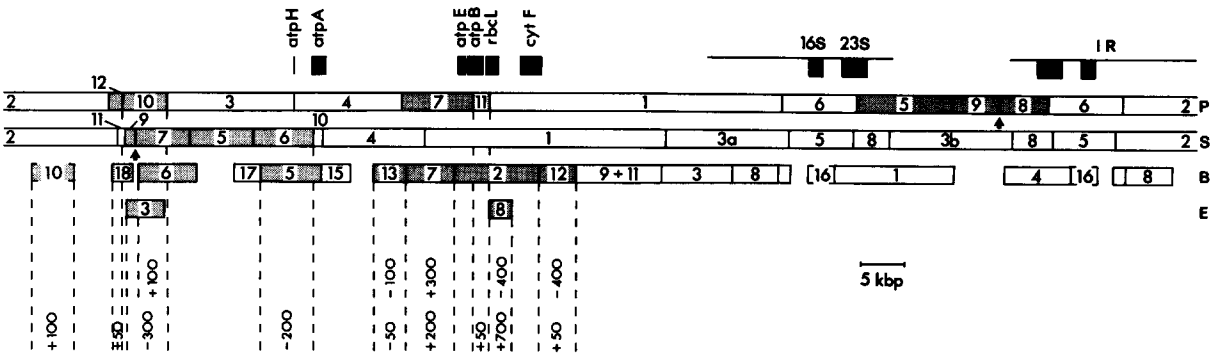

Fig. 6. Location of heterogeneity in the *Triticum*/*Aegilops* chloroplast genome. The serial order of the Pst and Sal fragments is shown in linear form with the S2/P2 limit of the inverted repeat at the ends. The positions of the larger Bam fragments and two of the Eco fragments are also shown. Fragment numbers are written in square brackets where the precise position is not known. The fragments which were measurably variable between the 11 different ctDNA types are shaded. The amount of DNA gained (+) or lost (–) relative to the *T. aestivum* ctDNA is calculated to the nearest 50 bp or 100 bp and the limits within which the deletion/insertion occurs are indicated by dotted lines. Arrows mark restriction endonuclease sites which are lost. The extent of the inverted repeat (IR) and the positions of the structural genes for 23S rRNA, 16S rRNA, RuBPCase large subunit (rbcL), cytochrome f (cyt F), and for the ATP- synthase subunits: α (atpA), β (atpB), ε (atpE) and the proton-translocating subunit (atpH) are also shown

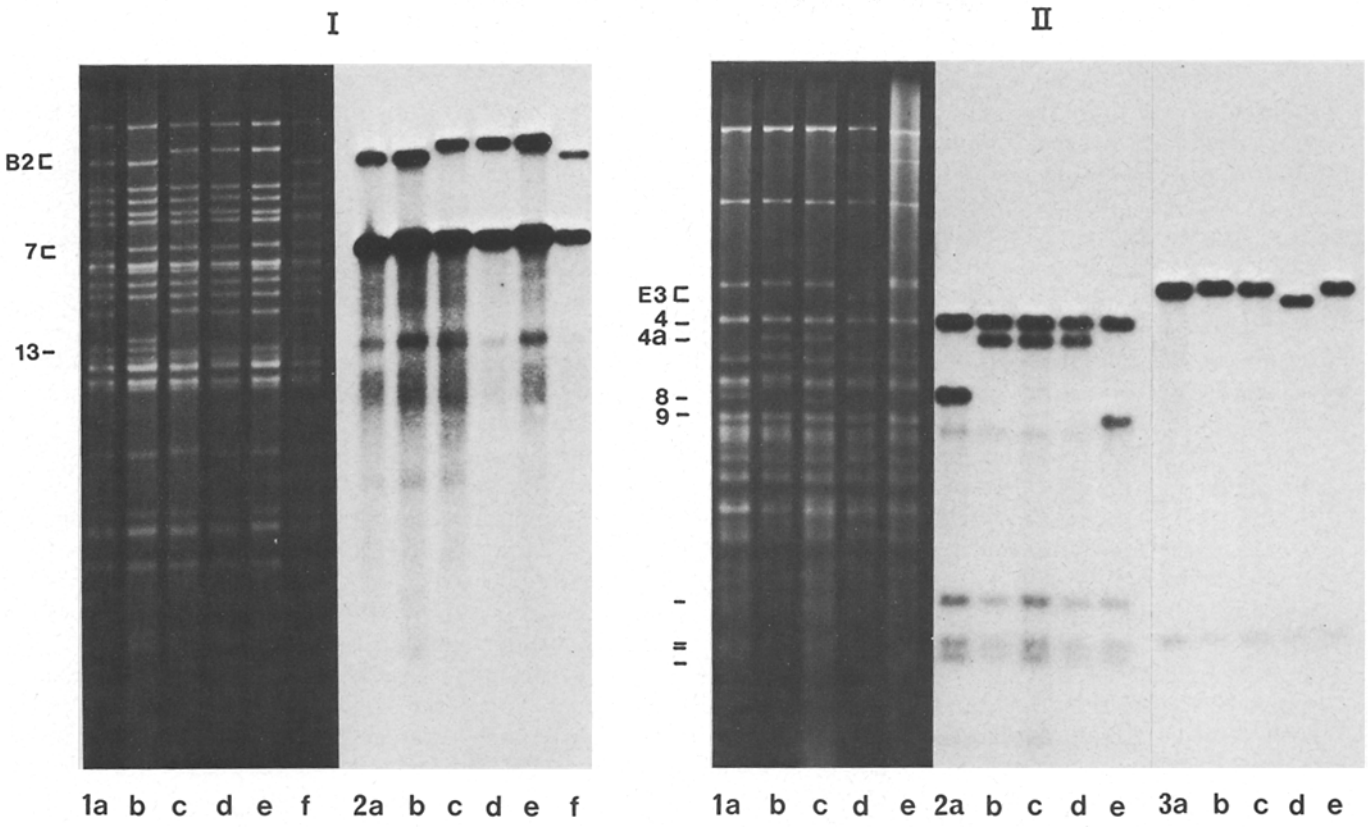

Fig. 7. Detection of deletions/insertions in different *Triticum/ Aegilops* ctDNA types. **I** (*1*) BamHI digests, **II** (*1*) EcoRI digests of ctDNA, fractionated by agarose gel electrophoresis. **I** (*a*) type B (*T. aestivum*) (*b*) type S (*Ae. speltoides* M), (*c*) type S^2 (*Ae. speltoides* S), (*d*) type S^b (*Ae. bicornis*), (*e*) type A^2 (*T. urartu*), (*f*) type D (*Ae. squarrosa*). **II** (*a*) type B (*T. aestivum*), (*b*) type S^b (*Ae. bicornis*), (*c*) type A^1 (*T. monococcum*), (*d*) type A (*T. boeoticum*), (*e*) type D (*Ae. squarrosa*). Hybridisation were with cloned, ^{32}P-labelled *T. aestivum* ctDNA fragments **I** (*2*) P7, **II** (*2*) B2 and **II** (*3*) P10

was previously wrongly identified as fragment E6 (Bowman et al. 1981) due to the similarity in size. Twenty-one of the 23 ctDNA fragments which were measurably variable between 11 ctDNA types are indicated by shading; the exact positions of E2a and E13 are not known.

There are many correlations in the fragment variations (listed in Table 1) which can be readily understood in terms of the location of those fragments on the chloroplast genome. The approximate extent of the size variation and the limits within which they occur are also indicated in Fig. 6. Because many of the variable fragments are relatively large (4–11 kbp) the size changes have been estimated only to the nearest 100 bp, or, in the case of the smaller fragments, to the nearest 50 bp. It will become clear that several of the correlated changes in fragment size represent DNA insertions or deletions, and that relative to the recognition sites for BamHI, EcoRI, PstI and SalGI, most of these appear to be clustered in two regions of the genome. In Fig. 6, the approximate amount of DNA gained (+) or lost (–) is indicated relative to the *T. aestivum* (type B) ctDNA map. In the text, changes in fragment size are also described relative to the *T. aestivum* ctDNA, as they are in Table 1, but the terms "insertion" and "deletion" are used on the assumption that ctDNA types A, B, D and S^2 are the more recent forms of the genome.

Many of the DNA alterations that were detected by Bam, Eco, Sal and Pst analysis are located in the region of the genome containing the fragments B2 and P7. Previous mapping experiments had suggested that all those Bam fragments which show 3 size variants (B2, B7, B12 and B13) map contiguously on the *T. aestivum* ctDNA (Fig. 6). The autoradiographs in Fig. 7 (Panel I) show that the alternative size variants of B2, B7 and B13 all hybridise with fragment P7 and therefore also map in the same region of the genome as the original *T. aestivum* ctDNA fragments. P7 does not overlap B12 and therefore cannot be used to map the B12 variants, but it is unlikely that these variants of B12 do not map as shown in Fig. 6. The comparative digests shown in Figs. 1, 2 and 5 indicated that the fragment B2 itself contains the site of a large deletion/insertion event. This event was manifested as a correlated variation in the size of fragments B2 and E8 (Table 1): in each

ctDNA type showing an approximately 700 bp increase in the size of B2 from 9.6 kbp to 10.3 kbp (Fig. 1), the 2.68 kbp fragment E8 has been replaced by the 3.4 kbp fragment E4a (Fig. 5). To confirm that this correlation does represent a deletion/insertion, ctDNA types showing each of the possible fragment variants from the B2/P7 region of the genome (Fig. 6) were digested with BamHI and EcoRI and hybridised with ^{32}P-labelled probes made from cloned *T. aestivum* ctDNA fragments B2 and P7. The data is shown in Fig. 7. In the autoradiograph illustrated in panel I (2), the relationship between the variants of B2 is established. In panel II (2) it is shown that in each ctDNA type showing variation in this region, B2 overlaps fragments E4, E4a, E8 and E9, plus some unnumbered fragments. In each type showing the 10.3 kbp variant of B2 (panel II (2) tracks b, c, d), the only change in the hybridising Eco fragments is the replacement of E8 by E4a. There is no associated loss of a 0.7 kbp Eco fragment, as would be expected if E4a were generated by the loss of an Eco site between E8 and an adjacent 0.7 kbp fragment. It is clear too, that the 0.4 kbp decrease in the size of B2 in type D ctDNA (track e) also represents a deletion in this region of the genome; E8 has in this case been replaced by an additional 2.3 kbp fragment, E9. (Two fragments which might have been involved in these DNA alterations did not hybridise to B2: the 4.55 kb fragment E2a in the type D ctDNA (track e), and the *T. monococcum* type ctDNA 2.65 kbp E8 fragment (track c)). Because the type D and type B ctDNAs are assumed to be the more recent, the size variation of fragments B2 and E8 can be interpreted as expressing deletions in this region of the genome of 0.7 kb in the B-type ctDNA of *T. aestivum* and of 1.2 kb in the D type ctDNA of *Ae. squarrosa.*

Smaller deletions or insertions in the same region of the genome are suggested by the correlated variations in the size of fragments B7 and P7. In the different DNA types, fragment B7 can vary from 5.5 kb in *T. aestivum* to 5.76 kbp or 5.85 kbp in the other types (Fig. 1, Table 1, Fig. 7). In the overlapping 8.1 kbp fragment P7, the increase in size alters the band mobility to coincide approximately with that of the 8.4 kb doublet P6 (Fig. 3). Therefore in the case of type B ctDNA, deletions of the order of 200 and 300 bp are indicated. It is not clear whether the change from the 5.76 kbp to the 5.85 kbp variant of B7 in type A ctDNA represents a deletion or an insertion. Further alterations of the DNA in this area of the genome are represented by the 3 variants of fragments B12 and B13 and by the increase of approximately 50 bp in the length of P11 in the S^2-type ctDNA of *Ae. speltoides* S.

The second region of the genome in which several DNA alterations have been detected is in the area containing P12, P10 and S7. In the A-type ctDNA of *T. boeoticum,* fragment P10 is reduced in length by approximately 300 bp, from 5.2 kb to 4.9 kb (Fig. 3, Table 1). A similar reduction is evident in the length of fragment E3. The autoradiograph in panel II (3) of Fig. 7 shows that E3 is the only Eco fragment hybridising with P10, which shows a corresponding decrease in size. Thus a deletion of 300 bp has occurred in the type A ctDNA. The accompanying disappearance of fragments S7 (6.1 kb) and S9 (1.2 kb) in the A-type ctDNA and the appearance of an additional S5 (7.2 kb) fragment (Fig. 4, track 1), indicates that the SalI recognition site between S7 and S9 has been lost, probably in the same deletion (Fig. 6). Two other alterations also map in this region of the genome. A deletion of approximately 100 bp is suggested by the change in length of B6 from 6.6 kb in *T. aestivum* to 6.7 kb and by the correlated change in length in the overlapping fragment S7 from 6.1 kb in *T. aestivum* to 6.2 kb in the other ctDNA types (Table 1). In the S^2 type ctDNA a probable 50 bp insertion is suggested by a 50 bp increase in the length of the overlapping fragments B18 and P12.

The remaining measurable fragment alterations are also found in the single-copy regions of the genome. In the D-type ctDNA, fragments B5 and S6 are both reduced in length by 200 bp, indicating a 200 bp deletion in the B5/S6 overlapping region (Fig. 6). The A-type ctDNAs all show an increase of approximately 100 bp in the length of B10 (Fig. 1, Table 1). It is not known whether this represents an insertion or deletion. The exact position of B10 within the P2/S2 overlap is also uncertain, so this alteration may map close to B18 and be included in the P12–S7 cluster, or, it may extend the "cluster" towards the inverted repeat region (Fig. 6). Finally, a second loss of recognition sequence was detected in the small single-copy region, between fragments P8 and P9 in the *Ae. longissima* (S^{12}-type) ctDNA. This was expressed as a duplication of the 11.0 kb fragment P5 and the disappearance of fragments P8 (5.6 kb) and P9 (5.3 kb).

No variations were detected in the inverted repeat region of the *Triticum/Aegilops* chloroplast genome.

Discussion

Chloroplast DNA variation and the classification of the cytoplasm

Analysis with the combination of restriction endonucleases BamHI, PstI, SalGI and EcoRI revealed interspecific and intraspecific variation between the ctDNAs and therefore between the cytoplasms of the several species of *Triticum* and *Aegilops* examined in this survey. The variation was extensive enough and yet simple enough for ctDNA analysis to be used as a basis for the classification of the *Triticum/Aegilops* cytoplasms. This is illustrated in Table 2, where the cyto-

Table 2. Comparison of two methods of cytoplasmic classification: restriction endonuclease analysis of ctDNA and analysis of the phenotypic effects of the cytoplasm in N-C hybrids

Species	Haploid genome formula	Cytoplasmic type		Detection of intraspecific variation[b]	
		ctDNA classification	Phenotypic effect classification[a]	ctDNA classification	Phenotypic effect classification[a]
T. aestivum	ABD	B	S	No (4)	No (4)
T. dicoccoides	AB	B	S	–	No (4)
T. timopheevi	AG	G	G	–	No (5)
T. araraticum	AG	G	G	–	–
Ae. speltoides M	S	S	S or G	Yes (6)	Yes (3)
Ae. speltoides S	S	S^2	S or G		
Ae. sharonensis	S^1	S^1	S^1	–	Yes (2)
Ae. searsii	S^s	S^1	–	–	–
Ae. longissima	S^1	S^{12}	S or S^{12}	–	Yes (2)
Ae. bicornis	S^b	S^b	S	–	–
T. boeoticum	A	A	A	Yes (3)	No (2)
T. monococcum	A	A^1	–		–
T. urartu	A	A^2	–		–
Ae. squarrosa	D	D	D	–	–

[a] Data from Tsunewaki et al. (1980)

[b] Presence or absence of detectable intraspecific variation is indicated by Yes or No respectively. The number of accessions/varieties analysed is given in brackets. –: species not analysed

plasmic classes recognised by ctDNA analysis and by the phenotypic effect analysis are compared. In general there is good agreement between the two methods, the ctDNA cytoplasmic classes fitting well into the framework of the cytoplasmic types recognised by Tsunewaki et al. (1980). The degree of resolution is similar; both methods are able to detect intraspecific variation, but the detail of the variation differs. One of the most obvious differences is the discrimination between the B and S type cytoplasms by ctDNA analysis. It is to avoid any confusion arising from these differences, that the ctDNA cytoplasmic classes have been denoted by the symbol for the nuclear genome of the diploid species from which that cytoplasm is derived. It is also worth noting that there is good agreement between the ctDNA classification and the accepted taxonomic classification of the species included in this survey. For example, the *Sitopsis* section of the genus *Aegilops* can be divided into two subsections: the *Truncata,* containing the *Ae. speltoides* types, and the *Emarginata,* containing *Ae. sharonensis, Ae. longissima* and *Ae. bicornis* (e.g. Feldman 1978). These two subsections are also separable by ctDNA analysis (Table 2). Also, there is clearly no strict demarcation between the ctDNA banding patterns of the *Triticum* and *Aegilops* species. This is consistent with the proposal that there is no real taxonomic distinction between the two genera, and that all these species could be included in the genus *Triticum* (e.g. Bowden 1959).

Among the diploid *Sitopsis* cytoplasms, that of *Ae. searsii* has not previously been classified, because the recognition of *Ae. searsii* as a species separate from *Ae. longissima* is relatively recent (Feldman and Kislev 1977). The ctDNA analysis indicates that the *Ae. searsii* cytoplasm belongs to the same group (S^1) as that of *Ae. sharonensis. Ae. sharonensis* itself, previously identified as belonging to the same group as *Ae. longissima*-N (Tsunewaki et al. 1980), is shown to belong to a separate cytoplasmic group. Intraspecific variation of the cytoplasm was revealed by ctDNA analysis of 6 populations of *Ae. speltoides.* Interestingly, five of the populations showed a common ctDNA type (S), even though they were collected from two geographically separate locations (two from Turkey and three from Israel). This suggests a general conservation of the *Ae. speltoides* cytoplasm, and yet, the *Ae. speltoides* S-type cytoplasm (S^2), also from Turkey, shows a unique ctDNA pattern. This observation is consistent with those of Tsunewaki et al. (1980) who also detected two cytoplasmic types among the three populations of *Ae. speltoides* which they studied (Table 2). The detection of intraspecific variation in ctDNA means that it is advisable to establish the ctDNA type representative of a given species by examining several populations. It is also obviously necessary to interpret with appropriate caution data drawn from only one population.

Three species, *T. boeoticum, T. monococcum,* and *T. urartu,* were included in the survey as examples of the 'A' type cytoplasm, and ctDNA analysis showed that the cytoplasms of all these species were different. Variation has not previously been detected among A-type cytoplasms, simply because only one representa-

tive, *T. boeoticum,* was used in the phenotypic effect analyses.

As expected, the cytoplasm of *Ae. squarrosa,* the only example of a D-type cytoplasm, was found to be unique.

In the diploid species ctDNA variation was common. In contrast, the polyploid species analysed, though few in number, did not show unique ctDNA types. The two tetraploids *T. timopheevi* and *T. araraticum* had identical ctDNA banding patterns and were classified as having cytoplasmic type G, in agreement with the classification of Tsunewaki et al. (1980). Chloroplast DNA analyses of *T. dicoccoides* and the four varieties of *T. aestivum* were also indistinguishable from each other, and these were classified as having type B cytoplasms. Previously, the *T. aestivum*/*T. dicoccoides* cytoplasm was classified, according to the similarity of its phenotypic effects, with cytoplasms of the *Sitopsis* section species in group S.

Recently and independently, Ogihara and Tsunewaki (1982) have similarly analysed the ctDNAs from 38 of their N–C hybrid lines. Consequently they have also reclassified the *T. aestivum* cytoplasm as belonging to group B. Analysis of the 38 cytoplasms revealed 10 distinct ctDNA types. Five of these are also described in this study: B, S, G, D and S^b, the remaining five are associated with cytoplasms which we did not analyse: D^2, C, C^u, M^t and M^o. Many of the band variants were the same as those listed in Table 1 but significantly, even the new band variants, diagnostic of these 5 new ctDNA types, represent DNA alterations in the same regions of the chloroplast genome as those illustrated in Fig. 6: another variant of B2 was found in types C, C^u and M^t; a larger (0.5 kbp) deletion was found in the E3/P10 region of type M^u and the Pst site between P10 and P12 was lost in one of the S-type ctDNAs; fragment B17 was lost by the disappearance of the Bam site between B17 and B5 in types M^o and C^u, and by an unexplained DNA alteration in type M^t. Therefore, in these two parallel studies, 16 ctDNA types have been found on analysis of 58 cytoplasms. The absence of detectable differences in ctDNAs from the same cytoplasm whether in the wild species, as described in this paper, or in N–C hybrid lines as described by Ogihara and Tsunewaki (1980), suggests that the substitution of a cytoplasm with an alien nucleus does not usually alter the ctDNA organisation. Interestingly, as a consequence of their ancestry, the variation detected between the 16 distinct ctDNA types resides in the varied combination of a relatively few DNA alterations which tend to occur in particular regions of the genome.

These characteristics of the *Triticum*/*Aegilops* ctDNA banding patterns and the convenience of the procedure, make restriction endonuclease analysis of ctDNA a useful tool for cytoplasmic classification. A comparative drawback of the phenotypic effect analysis is the need to stabilise a homogeneous nuclear genome in the N–C hybrids by at least 5 backcross generations before the phenotype can be analysed reliably.

Phenotypic significance of ctDNA banding patterns

The various cytoplasmic types show considerable variation in their levels of incompatibility with *T. aestivum* nuclear genes in N–C hybrid plants. Consequently the cytoplasms also differ remarkably in their effect on the hybrid plant phenotype, and can be classified on this basis. CtDNA analysis has revealed that the types of ctDNA associated with various cytoplasms are also distinctly different. There is, however, no correlation between any variation found in a given ctDNA banding pattern, and the phenotypic effects known to be associated with that cytoplasm. On the contrary, variant bands are often common to several ctDNA types (Table 1) which are associated with cytoplasms showing a spectrum of phenotypic effects (Tsunewaki et al. 1980). The D-type ctDNA, which differs greatly from the B-type ctDNA of *T. aestivum,* is associated with the cytoplasm of *Ae. squarrosa,* which causes no deleterious effects when combined with the *T. aestivum* nucleus in N–C hybrid plants. Conversely, the "compatible" D-type ctDNA also shows all those variant bands which are found in the G-type ctDNA: B6, B7, P7, P10, S7 and E3, and yet the G-type cytoplasm causes N–C hybrids to be male sterile (Tsunewaki et al. 1980). As a second example, the *T. boeoticum* cytoplasm is known to cause both winter killing and variegation in N–C hybrids, and in this survey *T. boeoticum* ctDNA was found to be unique in having a 0.3 kb deletion in the E3/P10 region of the genome. However, *Ae. umbellulata* cytoplasm also causes a similar variegation and its ctDNA does not show this deletion (Ogihara and Tsunewaki 1982). Also, a 0.5 kb deletion has been observed in this same region of the genome in *Ae. uniaristata* ctDNA (Ogihara and Tsunewaki 1982) a species whose cytoplasm does not cause variegation or winter killing.

While ctDNA analysis appears to be a promising method for classifying and identifying cytoplasmic types among *Triticum* and its related species, there are two reasons why correlations between changes in ctDNA banding patterns and cytoplasmic compatibility are rare. First, restriction enzyme analysis reveals only a small proportion of the variation in the ctDNA sequence but second and more important, much of the cytoplasmic incompatibility may be attributable to the mitochondrial genome. The evidence for this is particularly strong concerning male sterility (e.g. Kemble et al. 1980; Galun et al. 1982). The banding patterns of *Triticum* and *Aegilops* mitochondrial DNAs are rather complex to be useful for classification purposes (e.g.

Vedel et al. 1976). However, because mitochondrial and chloroplast genes are inherited maternally in these species (e.g. Vedel et al. 1981), identification of a cytoplasm by ctDNA analysis should still allow a prediction of cytoplasmic compatibility in crosses between bread-wheat and its wild relatives.

The inheritance of the B cytoplasm and the B genome

From their extensive studies of cytoplasmic classification, Tsunewaki et al. (1976) proposed a scheme for the inheritance of the cytoplasm from the diploid to the polyploid species of *Triticum* and *Aegilops*. The cytoplasm of the hexaploid *T. aestivum* was found to be similar to the S-type cytoplasm of the *Sitopsis* section of the genus *Aegilops*. Therefore, a *Sitopsis* group diploid was suggested as the probable maternal parent in the original cross between the A-genome and S-genome diploids which produced the ancestor of the wild AB-genome tetraploid *T. dicoccoides* (Tsunewaki et al. 1976; Suemoto 1978). Thus the donor of the cytoplasm to hexaploid *T. aestivum* was also the donor of some, if not all, of the chromosomes comprising its B genome. Studies of nuclear genes and their products have suggested several particular diploid *Aegilops* species from the *Sitopsis* group as possible B genome donors:

Ae. speltoides (Sarkar and Stebbins 1956; Riley et al. 1958; Jaaska 1978), *Ae. searsii* (Feldman 1978), *Ae. bicornis* (Sears 1956), *Ae. longissima* (Vittozzi and Silano 1976; Gerlach 1978), *Ae. sharonensis* (Kushnir and Halloran 1982) and *T. urartu* (Johnson 1975).

We therefore chose representatives of these species for ctDNA analysis. Six populations of *Ae. speltoides* were included because, to date, *Ae. speltoides* is the species for which the most positive evidence has been accumulated, through studies on plant morphology (Sarkar and Stebbins 1956), chromosome pairing (Riley et al. 1958) ADH-B isoenzymes (Jaaska 1978) cytoplasmic compatibility with *T. aestivum* (Tsunewaki et al. 1976; Suemoto 1978) and isoelectric focussing of ribulose bisphosphate carboxylase (RuBPCase) (e.g. Chen et al. 1975).

For purposes of phylogeny, analysis of a multicopy genome such as ctDNA has several advantages over analysis of nuclear genes and their products because it is less prone to DNA alterations through mutation, recombination and introgression. It will however detect only one donor if the origin of the B genome is polyphyletic, and the possibility of intraspecific variation of ctDNA must also be borne in mind.

Restriction endonuclease analysis of the ctDNAs from the many species included in this survey showed that *T. dicoccoides* alone belonged to the same cytoplasmic group, B, as that of *T. aestivum*. Therefore, the donor of the cytoplasm to *T. aestivum* was not detected among the diploid species or *Ae. speltoides* populations represented here. Our results support the conclusions of Vedel et al. (1978) and Kimber and Athwal (1972) who, on the basis of mtDNA banding patterns and chromosome pairing studies respectively, discounted *Ae. speltoides* as a possible donor of the B genome. In their analysis of alloplasmic lines, Ogihara and Tsunewaki (1982) found that the ctDNA banding pattern associated with *Ae. longissima* cytoplasm was different from that of the *Ae. longissima* population described in this paper. This is consistent with the observation of intraspecific variation between *Ae. longissima* cytoplasms by Tsunewaki et al. (1980) (Table 2). Moreover, the *Ae. longissima* ctDNA banding patterns described by Ogihara and Tsunewaki (1982) were identical with those of *T. aestivum* and *T. dicoccum*. Therefore, it appears that the donor of the cytoplasm and the B genome to the hexaploid wheat *T. aestivum* was probably a population of the *Sitopsis* group diploid *Ae. longissima*.

The ctDNA analyses described in this paper support several other suggestions concerning wheat ancestry: that *T. dicoccum*, the tetraploid progenitor of *T. aestivum*, was the cultivated from of the wild tetraploid *T. dicoccoides* (Percival 1921) and similarly that *T. timopheevi* is the cultivated form of the wild tetraploid *T. araraticum* (e.g. Maan 1975). It has also been suggested that the *Timopheevi* wheat group diverged from the Emmer/Dinkel group, either at the diploid level, originating from modified forms of *Ae. speltoides* (Tsunewaki et al. 1980) or at the tetraploid level (Maan 1975; Tanaka 1978). The extreme similarity between the ctDNA banding patterns of *T. dicoccoides* and *T. araraticum* (Table 1) certainly suggests that the cytoplasms of the two tetraploids are closely related. However, the close similarity between the *Timopheevi*/*Araraticum* G-type ctDNA and the *Ae. speltoides* S-type ctDNA (Fig. 1 and Table 1) implies that the divergence may have occurred at the diploid level, and that the cytoplasms of the *Timopheevi* wheats and the Emmer wheats originated from populations of the *Sitopsis* diploids *Ae. speltoides* and *Ae. longissima* respectively.

T. urartu, although once proposed as a B-genome donor (Johnson 1975) is now recognised as an A-genome diploid (e.g. Dvorak et al. 1979). Accordingly, ctDNA analysis has identified it as belonging to cytoplasmic type A. Of the two other A-type diploids we have analysed, *T. boeoticum* ssp. *aegilopoides* has been considered as the possible wild form of the cultivated diploid *T. monococcum*, (e.g. Percival 1921). The ctDNA banding patterns shown in Fig. 5 suggest that neither of the populations of the A-type diploids *T. urartu* or *T. boeoticum* that we examined fulfilled this role. This is easily understood, as the diploid cultivar *T. monococcum* was evidently domesticated

repeatedly from the wild species by the early agricultural tribes (Johnson 1975).

Among the small number of polyploid species included in this survey, none showed unique ctDNA fragments. All of the band variation detectable in the *Triticum/Aegilops* ctDNA was present in the diploid species. This general observation also extends to the parallel study of Ogihara and Tsunewaki (1982), who analysed 25 polyploid species. Thus all the DNA alterations which characterise the several distinct ctDNA types are found in the ctDNAs of the diploid species. This data supports the suggestion of Maan (1975) and Tsunewaki et al. (1980) that cytoplasmic variability among *Triticum* and *Aegilops* species antedated allopolyploidy.

We have not used the similarity in ctDNA fragment patterns to estimate the divergence between ctDNA types in any detail, because we did not consider there to be sufficient data for this purpose. Scoring each DNA type according to the rarity of its banding patterns does however reveal that among the 11 ctDNA types that were detected, four (B, S^2, A and D) are probably of relatively recent origin. This makes it possible in many cases to deduce whether the fragment variants found in those ctDNA types are likely to be the result of deletions or insertions.

Location of heterogeneity in the Triticum/Aegilops chloroplast genome

Comparing the 11 variants of the *Triticum/Aegilops* chloroplast genome has revealed an interesting pattern of heterogeneity. There are several DNA deletions and insertions which map close to known genes for chloroplast proteins and which are therefore potentially useful for studying the in vivo regulation of chloroplast genes. It is clear from Fig. 6 that the structural genes for the RuBPCase large subunit (LS), (Bowman et al. 1981), cytochrome f (Willey and Gray, unpublished results) and the ATP synthase subunits β and ε (Howe et al. 1982b) are all located within the particularly variable region of the genome contained in, and surrounded by, fragments B2 and P7. If it is assumed that the D-type and B-type are derived forms of the chloroplast genome, then it appears that two large deletions have occurred in a very specific region between the LS gene and the cytochrome f gene. It is known that in the B-type ctDNA of *T. aestivum*, the coding region of the LS gene extends from the right-hand border of fragment P11, to about 1.0 kbp into the 2.7 kbp fragment E8 (Koller et al. 1982); therefore, in *T. aestivum* ctDNA, a $\sim$ 700 bp deletion is likely to have occurred within the 1.7 kb segment downstream of the LS gene. If this logic is extended to the type-D ctDNA, then a larger deletion of $\sim$ 1,200 bp has occurred to reduce the size of "E8" to 2.3 kbp. It is significant that in EM analyses of heteroduplexes between ctDNA of *Zea mays* and *T. aestivum* from this region, the formation of heterologous loops implies that the homology between the two begins to break down downstream of the protein-coding segment of the LS gene (Koller et al. 1982). The same is true if DNA sequences of the LS gene and its flanking sequences are compared between *Zea mays* (McIntosh et al. 1980), *Spinacia oleracea* (Zurawski et al. 1981) and *T. aestivum* (Saul and Dyer, unpublished results). An insertion of approximately 50 bp was detected in the P11 fragment of S^2-type ctDNA. Fragment P11 contains the 5′ region of the LS gene separated from the 5′ two-thirds of the ATP synthase β subunit gene (on the opposite strand) by a 350 bp "spacer" region which contains the promotors for both the LS gene and the β subunit gene (Koller et al. 1982). Therefore, the 50 bp insertion may affect the transcription of either of these genes from the S^2 type ctDNA of *Ae. speltoides* S. The remainder of the β subunit gene and the ε subunit gene do not overlap the variable fragment B7. The gene for the proton-translocating subunit of ATP synthase also maps on a variable fragment, S6 (Howe et al. 1982a) which shows a $\sim$ 200 bp deletion in the D-type ctDNA. However, this fragment is so large that the deletion may map up to 4 kb away from the gene.

Observations such as these suggest that a study of DNA transcription using the B, A, S^2, D and S cytoplasms, coupled with a more detailed study of DNA sequence and organisation in the ctDNA types associated with those cytoplasms, should prove fruitful.

The data presented in this paper agrees with current thinking on conservation and rearrangement of the chloroplast genome during evolution. The close similarity of banding patterns, the correlations in fragment variation relative to the *T. aestivum* ctDNA map, and the mapping of particularly variant regions by cross hybridisation, indicate that there have been no large-scale rearrangements of the fragments themselves during evolution of the *Triticum* and *Aegilops* ctDNAs. All of the fragment variation was detected in the single copy regions of the DNA. In other studies where closely-related ctDNAs containing inverted repeats have been compared, a similar conservation of serial fragment order has also been observed, and the majority of the variant fragments have also been located in the single-copy regions of the genome (e.g. Gordon et al. 1982). On the few occasions that variant fragments have been detected in the inverted repeat regions, a variation in one half of the repeat is usually complemented in the other (e.g. Gordon et al. 1982). An exception to this generalisation has however recently been observed in the ctDNAs of the genus *Nicotiana* (Shen et al. 1982). If ctDNAs from plants such as *Pisum sativum* and *Vicia faba* are compared, in

which the inverted repeat region has not been conserved, then extensive DNA rearrangements are detectable (Palmer and Thompson 1982). Therefore it is believed that (a) a mechanism exists for conserving the homology between the two halves of the inverted repeat, and (b) the presence of the inverted repeat itself is associated with a more stable form of the chloroplast genome.

The apparent clustering of several DNA alterations, such as those illustrated in Fig. 6, has been interpreted in several studies of ctDNA organisation as representing "hotspots" of DNA rearrangement (e.g. Kung et al. 1982). It does seem that within the limits of sampling used in these experiments, DNA insertions and deletions have accumulated during evolution in certain regions of the *Triticum/Aegilops* chloroplast genome. The data of Ogihara and Tsunewaki (1982) reporting additional alterations in these same regions further strengthens this view. Examination of different ctDNAs from *Nicotiana* and *Oenothera* also reveals hotspots in the large single-copy region of the genome, but the alterations are clustered differently. In *Nicotiana* ctDNA the variation maps near the border of one of the inverted repeats (Kung et al. 1982), while in *Oenothera* the variation is concentrated towards both inverted repeat regions, and the centre of the large single-copy region, surrounding the (LS) gene, is found to be particularly invariant (Gordon et al. 1982).

It appears that the variable regions of the ctDNA detected in *Triticum* and *Aegilops* species and in particular the regions in fragments B2/E8 and P10/E3 showing the larger deletions, may be involved in two gross rearrangements which are suggested to have occurred during the evolution of ctDNAs on a wider scale (Palmer and Thompson 1982). Divergence of the legume family has involved a large 50 kbp inversion relative to the ancestral chloroplast genome, most obvious in the mung bean *Vigna radiata.* Divergence of the monocots (e.g. *Zea mays*) has apparently involved a smaller inversion (Palmer and Thompson 1982). Cross hybridisation of ctDNA fragments containing protein-coding genes from spinach, pea and wheat suggests that in *T. aestivum* ctDNA one end of the 50 kbp inversion lies between the LS gene and cytochrome f gene, where the 700 kb and 1,200 kb deletions were detected, and the other lies in fragment S2, close to the inverted repeat. Similarly, one end of the smaller inversion is thought to map in the fragment B15, which contains part of the gene for the ATP synthase α subunit (Fig. 6) (Howe et al. 1983), while the other end probably maps to the right of B18 (T. A. Dyer, unpublished results) where deletions of approximately 300 bp (Fig. 6) and 500 bp (Ogihara and Tsunewaki 1982) have been detected. It is possible therefore, that these variable regions of the chloroplast genome contain sequences which have allowed DNA recombination and rearrangement to occur.

For this examination of ctDNA organisation we chose species from the genera *Triticum* and *Aegilops*, whose phylogeny has been studied over the past three decades. The relatively simple pattern of DNA heterogeneity that was revealed by restriction endonuclease analysis is presumably a consequence of the close phylogenetic relationship between these species. As we have described in this paper, such simplicity makes ctDNA analysis a useful tool for the identification and classification of the cytoplasm, and hence also for phylogenetic studies. It is however impossible to attach any phenotypic significance to the ctDNA variation observed at this level. Even though the different cytoplasms chosen for analysis were already known to be associated with specific phenotypic effects in N–C hybrids, and were then also found to contain distinctly different types of ctDNA, no correlation was observed between ctDNA organisation and the phenotypic effects of the cytoplasm. To understand the involvement of chloroplast genes in the production of the plant phenotype in these species, more detailed studies are needed. Appropriate analysis of species containing distinct ctDNA types would reveal whether there are any specific DNA alterations which affect the transcription of the chloroplast genome in vivo.

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