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Restriction fragment patterns of chloroplast and mitochondrial DNA of *Dasypyrum villosum* (L.) candargy and wheats

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Abstract. To elucidate the phylogenetic relationships and cytoplasmic types, restriction endonuclease fragment patterns of chloroplast (cp) and mitochondrial (mt) DNAs isolated from two different accessions of *Dasypyrum villosum* (L.) candargy were compared with those of tetraploid wheat (*Triticum durum* Desf., PI265007), hexaploid wheat (*Triticum aestivum* L., cv Chinese Spring), *Aegilops longissimum* (S. and M., in Muschli) Bowden and *Hordeum vulgare* L. *T. aestivum* and *T. durum* had identical restriction patterns for their cp and mtDNAs in digestions with four different enzymes. Likewise, no differences were found between the restriction fragment patterns of two accessions of *D. villosum*. But, there were distinct differences in chloroplast and mitochondrial DNA restriction fragment patterns between *D. villosum* and tetraploid and hexaploid wheats. *A. longissimum* (G609) showed a similar pattern to those wheats for *Pst*I digestion of cpDNA. Organellar DNA from *Hordeum vulgare* (cv Himalaya) showed a distinctly different restriction pattern from those of wheat and *D. villosum*. These results suggest that *D. villosum* is unlikely to be the donor of cytoplasm to wheats, and its cytoplasmic organelles were also different from those of *A. longissimum*.

Key words: *Triticum aestivum* – *T. durum* – *Aegilops longissimum* – *Dasypyrum villosum* – Endonuclease – Cytoplasm donor

Introduction

Evolution of species in the genus *Triticum* has been shown to involve multiple hybridization and polyploidization events among related diploid species (Kimber and Sears 1987). The ancestral diploids of the polyploid *Triticum* species have been inferred by genome analysis, biochemical (isozyme and storage protein) determinations, and morphological comparisons. *T. durum* is an allotetraploid containing homoeologous A and B genomes, and *T. aestivum* is an allohexaploid containing three homoeologous genomes, A, B, and D. The diploid ancestors that contributed the A and D genomes are known to be *Triticum monococcum* and *Triticum tauschii* (syn. *Aegilops squarrosa*), respectively (Kerby and Kuspira 1987; Kimber and Sears 1987). However, the source of the B genome remains unclear and controversial. The general consensus is that one or more of the S genome species within the section *Sitopsis* donated the B genome to cultivated wheats (Talbert et al. 1991).

Dasypyrum villosum (L.) Candargy (syn. *Triticum villosum* and *Haynaldia villosa* Schur, $2n=14$, VV), is an annual allogamous grass species of the tribe *Triticeae*, family *Gramineae*. The relationship between *D. villosum* and wheat has been explored in various ways. Montebove et al. (1987) found homoeological relationships between the chromosomes of *D. villosum* and hexaploid wheats through determination of loci and sites for isozymes and storage proteins. By contrast, Friebe et al. (1987) reported that distinct differences occurred in C-banding patterns between the chromosomes of *D. villosum* and hexaploid wheat. Nevertheless, *D. villosum* is cross-compatible with tetraploid and hexaploid wheats (Jan et al. 1986) and has been recognized as a potential source of useful genes for improving wheats (Qualset et al. 1981, 1984).

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Analyses of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) have been useful in elucidating the phylogenetic relationship in many plant taxa (Bowman et al. 1983; Palmer et al. 1983, 1988; Terachi et al. 1986, 1990; Doebley 1987; Palmer 1987). Chloroplast DNA was isolated from 35 wheat and *Aegilops* species and the chloroplast genome types were determined from their restriction fragment patterns. The 35 species (42 accessions) investigated were grouped into 16 chloroplast genome types by Ogihara and Tsunewaki 1988). However, *A. longissimum* and *D. villosum* were not included in the material they examined. Variation of cpDNA between species of *Triticum* and *Aegilops* was also analyzed by Bowman et al. (1983) and cpDNAs of *A. longissimum* and three other *Aegilops* species were found to be different from those of *T. aestivum* and *T. dicoccoides*. The latter two species had identical cpDNA patterns. However, *D. villosum* was not included in this study.

Terachi et al. (1990) performed restriction endonuclease analysis of mtDNAs from polyploid wheats and their related ancestral species, including *Aegilops* species. The mitochondrial genomes were divided into seven groups. However, the cytoplasm donor of the tetraploid-hexaploid wheat group could not be determined, and the mt genome from *D. villosum* was not analyzed.

The objectives of the present study were (1) to establish an efficient method to simultaneously extract cpDNA and mtDNA, especially from *D. villosum* and *Ae. longissimum*; isolation of DNA from seedling leaves of both species is difficult, and (2) to compare the cpDNA and mtDNA restriction fragment patterns of *D. villosum* with those of wheats and determine the possible relationships between the species.

Materials and methods

The materials tested included two accessions of *D. villosum*, collection A from Yugoslavia and collection B from Italy, *T. aestivum* (cv Chinese Spring), *T. durum* (PI265007), *A. longissimum* (G609), and *Hordeum vulgare* (cv Himalaya).

Isolation of cpDNA and mtDNA

DNA was isolated using a procedure modified from those of Mourad and Polacco (1989), Kolondner and Tewari (1975) and Ogihara and Tsunewaki (personal communication 1989). Seeds were planted in pots, and seedlings were grown in a greenhouse or a growth chamber. Two-week-old seedlings were transferred to darkness for 36 h. Leaves were harvested and rinsed with distilled water. In a cold room (4 °C), 30–40 g of leaves were cut into 1-cm sections and placed into a pre-chilled Waring blender. Six volumes of buffer I (0.44 M mannitol, 50 m*M* Tris HCl, pH 8.0, 5 m*M* EDTA, 1 m*M* β-mercaptoethanol, 0.1% BSA) were added to the cut leaves and homogenized using three pulses of 3 s each at the highest setting. The homogenate was filtered through seven layers of cheesecloth and four layers of miracloth and centrifuged at 1,000 g for 7 min (Sorvall GSA rotor, 2,500 rpm).

The supernatant was used for mtDNA isolation. The pellet containing chloroplasts was resuspended with a brush in 10 ml of buffer II (0.44 *M* mannitol, 50 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA). The suspension was transferred to a 50-ml tube and centrifuged at 1,000 g for 7 min (Sorvall SS-34, 3,250 rpm). The pellet was resuspended in 10 ml of buffer II and kept on ice. The supernatant containing mtDNA was centrifuged for 10 min at 1,000 g twice to remove residual chloroplasts and at 11,000 g once. The pellet containing mitochondria was then resuspended in 10 ml of buffer II.

Both chloroplast and mitochondrial suspensions were treated with DNase I (200 µg/ml) in 20 m*M* of $MgCl_2$ at 0 °C for 1 h. The enzyme reactions were stopped by adding 2 vol of buffer III (0.44 *M* mannitol, 50 m*M* Tris HCl, pH 8, 150 m*M* EDTA). The chloroplast suspension was centrifuged at 1,200 g for 10 min, and the pellet was suspended in 10 ml of buffer IV (0.44 *M* mannitol, 50 m*M* Tris HCl, pH 8, 20 m*M* EDTA). The above washing step was repeated eight times. The mitochondrial suspension was centrifuged at 12,000 g for 10 min. The supernatant was then centrifuged at 11,000 g for 10 min to collect the mitochondria. The mitochondrial pellet was resuspended in 5 ml of buffer IV, layered onto 20 ml of fresh buffer V (0.6 *M* sucrose, 1 m*M* Tris, pH 7.2, 20 m*M* EDTA), and centrifuged at 11,000 g for 20 min. The pellet was resuspended in 10 ml of buffer IV and centrifuged at 11,000 g for 10 min. The washing step was repeated.

The chloroplast and mitochondrial pellets were resuspended separately in 2 ml of buffer IV. Proteinase K (1 mg/tube) was added, and the mixture was incubated at room temperature for 10 min. Then 10% sodium lauroylsarcosinate was added (0.5 ml/tube), and the sample was incubated at 37 °C for 1 h in a water bath. Organelle DNAs were collected by isopycnic ultracentrifugation in a CsCl-ethidium bromide gradient, with n-butanol extraction and ethanol precipitation. The DNA was dissolved in 20–100 µl of TE buffer for restriction enzyme digestion.

Digestion of cpDNA and mtDNA with restriction enzymes

Aliquots of DNAs (1–2 µg) were digested with *Bam*HI, *Pst*I, *Hin*dIII, and *Sal*I according to the recommendation of the suppliers (Promega and Sigma). These five enzymes were among the 13 used by Terachi et al. (1990). The reactions were terminated by adding 5 µl of 10 X loading dye (2.5% bromophenol blue, 0.25% xylene cyanol, and 25% Ficoll, type 400).

Agarose gel electrophoresis

The digested cpDNAs and mtDNAs were loaded onto 0.8% and 1.0% agarose gels, respectively. Electrophoresis was carried out in TBE buffer (100 m*M* Tris, 100 m*M* boric acid, and 2 m*M* EDTA, pH 8) at 1.22 V/cm for 20–30 h at room temperature (BRL Horizontal, Model 14). The gel was stained with 0.5 µg/ml of ethidium bromide for 30 min and photographed with Polaroid 667 or Polaroid 665 P/N film on a UV transilluminator.

Results

Restriction fragment patterns from *Hin*dIII-digested cpDNA from the two accessions of *D. villosum* were identical, suggesting that there are no gross differences between the collections (Fig. 1). Likewise, no differences were detected between the *Hin*dIII restriction digest patterns of cpDNA from tetra- and hexaploid wheats

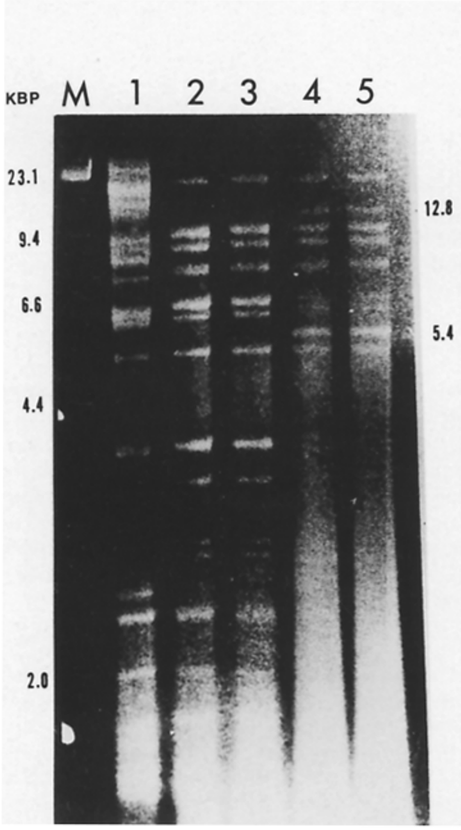

Fig. 1. Restriction fragment patterns of *Hin*dIII-digested cpDNA from *H. vulgare* (*lane 1*), *T. durum* (*lane 2*), *T. aestivum* (*lane 3*), *D. villosum* collection A (*lane 4*), and *D. villosum* collection B (*lane 5*)

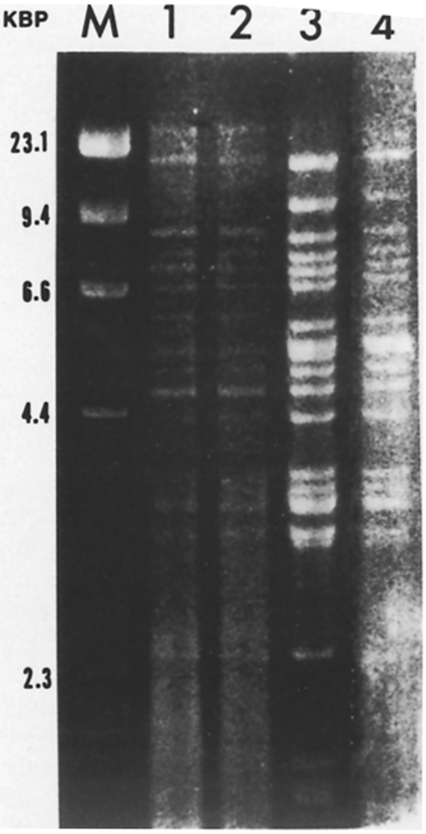

Fig. 2. Restriction fragment patterns of *Bam*HI-digested cpDNA from *D. villosum* collection A (*lane 1*), *D. villosum* collection B (*lane 2*), *T. aestivum* (*lane 3*), and *T. durum* (*lane 4*)

(Fig. 1, lanes 2,3), in agreement with previous reports (Kerby and Kuspira 1987; Kimber and Sears 1987). By contrast, *D. villosum* differed from the two wheats in the *Hin*dIII-digested cpDNA patterns. *D. villosum* had extra fragments of sizes 12.8 and 5.4 kb, but lacked seven smaller fragments at positions corresponding to 3.5, 3.2, 2.7, 2.6, 2.3, 2.0 and 1.8 kb, indicating that *D. villosum* is unlikely to be the maternal ancestor of the wheats. *H. vulgare* (cv Himalaya), analyzed as a reference species, had a *Hin*dIII-digested cpDNA pattern (lane 1) distinctly different from both wheats and from *D. villosum*.

Restriction patterns of *Bam*HI-digested cpDNA from wheats and *D. villosum* also revealed differences. The latter lacked one fragment at 12.9 kb and several smaller fragments, but contained an additional band longer than 23 kb (Fig. 2). *T. durum* had a restriction pattern that was indistinguishable from that of *T. aestivum* and the two collections of *D. villosum* showed identical patterns. The *Pst*I-digested fragment pattern of *D. villosum* was also different from that of wheat (Fig. 3). In particular, two bands at 12.9 kb and 5.6 kb were missing in *D. villosum* cpDNA.

*Sal*I-digested cpDNA showed no pattern differences between the two accessions of *D. villosum* or between tetraploid and hexaploid wheats. However, pattern differences were observed between *D. villosum* and wheats. An ideogram summarizing the results from the four restriction enzyme digestions is shown in Fig. 4. A coefficient of similarity (S) was calculated as the proportion of fragments (from the four endonucleases) common to both *D. villosum* and hexaploid wheat divided by the total number of observed fragments from both species. The coefficient of similarity was 24%, suggesting that *D. villosum* has a cpDNA that is more distantly related to wheat than any of the other 35 wheat and *Aegilops* species tested by Ogihara and Tsunewaki (1988).

Restriction fragment patterns of mtDNA from *Bam*HI digests produced many more fragments than those of cpDNA, as expected because of the larger genome size. Results from mtDNA digests were consistent with those from cpDNA and can be summarized as follows:

(1) The two accessions of *D. villosum* produced identical mtDNA restriction patterns. Likewise, mtDNA re-

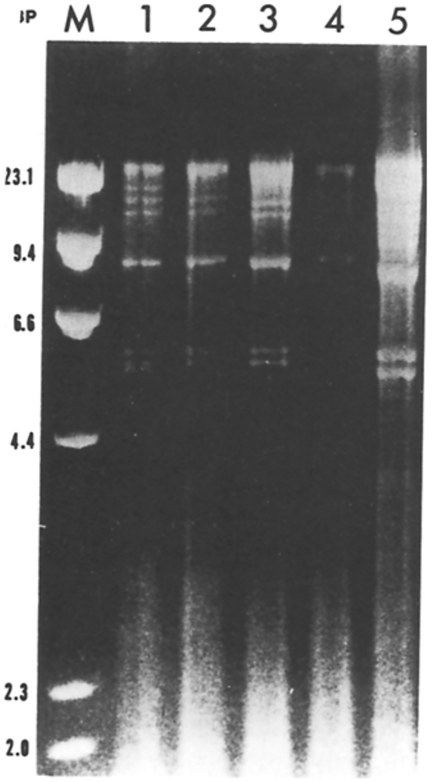

Fig. 3. Restriction fragment patterns of cpDNA-digested with endonuclease *Pst*I from *T. aestivum* (*lane 1*), *D. villosum* collection A (*lane 2*), *D. villosum* collection B (*lane 3*), *A. longissimum* (*lane 4*), and *H. vulgare* (*lane 5*)

striction patterns from *T. durum* and *T. aestivum* were identical.

(2) The mtDNA restriction patterns of *D. villosum* were consistently different from those of wheat.

Discussion

The linear array of genes in cpDNA is known to be highly conserved among evolutionarily distant species (Ogihara and Tsunewaki 1988). Analysis of variation in restriction fragments of cpDNA can provide valuable information concerning the cytoplasmic relationships among species that may not be apparent using other approaches, such as chromosome banding, crossability, or morphological traits. Mitochondrial genomes tend to show variability even among different populations of the same species or among accessions of the same species (Briman 1987; Palmer et al. 1988). Thus, meaningful comparisons among species are not always possible. Nevertheless, restriction fragment patterns of mtDNA have been useful for comparing wheats and their putative diploid ancestors (Terachi et al. 1990) as well as sorghum species at various ploidy levels (Lee and Liang 1989).

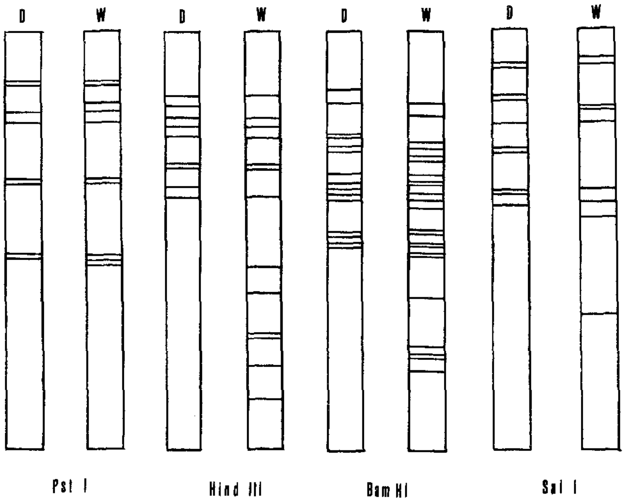

Fig. 4. Ideogram of restriction fragment patterns of cpDNA digested with endonucleases *Pst*I, *Hin*dIII, *Bam*HI, and *Sal*I, for *D. villosum* (*D*) and wheats, (*W*) *T. aestivum* and *T. durum*

Moreover, information from mtDNA analysis is especially useful when considered along with evidence derived from other experiments.

The extraction procedure was efficient in simultaneously obtaining cpDNA and mtDNA from each species, which saves time, material, and labor. The procedure not only works well for hexaploid wheat, durum wheat, and barley, but also for narrow-leafed *D. villosa* and *Ae. longissimum.*

Our results using cpDNA and mtDNA analyses suggest that *D. villosum* is unlikely to be the cytoplasm donor of wheat. This conclusion is based on the finding that the restriction patterns of *D. villosum* and wheats, observed with several endonucleases, were different. The coefficient of similarity for cpDNA was only 24% with 23 fragments in common from a total of 97 recognizable fragments. We used two different collections of *D. villosum* and were unable to detect any differences between them in RFLP patterns using four different enzymes and two organelle DNAs.

The cp- and mt-DNA restriction patterns were the same for *T. durum* (PI 265007) and *T. aestivum* (cv Chinese Spring) and the two wheats apparently have the same cytoplasmic organelles. This confirms previous reports (Ogihara and Tsunewaki 1982, 1988; Kerby and Kuspira 1987; Kimber and Sears 1987). *A. longissimum* had a cpDNA fragment pattern similar, but not identical, to that of wheats, a result parallel to that of Ogihara and Tsunewaki (1982) but different from that of Bowman et al. (1983). Because intra-specific variation is known to occur in chloroplast genomes (Harris and Ingram 1991), different accessions used in various experiments may lead to different results. This, if true, means that caution must be made in interpreting results.

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