Polymerase chain reaction-single strand conformational polymorphism analysis of intra- and interspecific variations in organellar DNA regions of *Aegilops mutica* and related species

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In order to study the phylogeny of *Aegilops mutica* in the genera of *Triticum* and *Aegilops*, variations in chloroplast and mitochondrial DNA regions were investigated by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis. Nine lines, each of *Ae. mutica* and *Ae. speltoides*, were studied together with nine other *Triticum* and *Aegilops* species, including *T. aestivum*. By analyzing 9.7-kb chloroplast and 13.1-kb mitochondrial DNA regions, a total of 268 bands were detected, of which 176 (65.7%) showed variation within and/or between species. The level of intraspecific variation of *Ae. mutica* was lower than that of *Ae. speltoides*. The low level of the intraspecific variation of *Ae. mutica* was contrary to the expectation from previous studies on morphological and cytological characters. In the phylogenetic trees based on SSCP, *Ae. mutica*, *Ae. speltoides* and the other four species of the section Sitopsis (the subsection Emarginata) were separated into three different clusters. In addition, *T. aestivum* was included in the cluster of *Ae. speltoides* in the phylogenetic trees. This result suggests that *Ae. speltoides* is the cytoplasmic donor of common wheat.

INTRODUCTION

The interspecific variation in organellar DNAs of *Triticum* and *Aegilops* species has been studied to determine the phylogenetic relationship among the species and to identify the cytoplasmic (B genome) donor of polyploid wheats (Ogihara and Tsunewaki, 1982, 1988; Tsunewaki and Ogihara, 1983; Bowman et al., 1983; Terachi and Tsunewaki, 1986; Terachi et al., 1990). The classification based on molecular variations is generally consistent with that obtained by classical genome analyses (Kihara, 1954; Kihara and Tanaka, 1970). In contrast with the studies on phylogenetic relationship, the intraspecific variation of *Triticum* and *Aegilops* species has not been well analyzed (Bowman et al., 1983; Breiman, 1987; Mori et al., 1988; Miyashita et al., 1994). These studies have shown that the level of intraspecific variation of organellar DNAs in *Triticum* and *Aegilops* species is low.

In the studies of inter- and intraspecific variation in the organellar DNAs of *Triticum* and *Aegilops* species, RFLP analysis has been most frequently used. Although this

technique can be applied to a large number of samples, it can reveal only a small proportion of the DNA variations. In this study, a new method, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) method, was employed to investigate variations in both chloroplast and mitochondrial DNAs. This technique consists of a sequence of simple procedures, i. e., PCR amplification, digestion of PCR product, if necessary, and electrophoresis on non-denaturing acrylamide gel. Variations are detected as mobility differences in the gel due to the secondary structure of single-stranded PCR products (Orita et al., 1989). PCR-SSCP can also analyze a large number of samples, but has a high sensitivity to detect almost all types of mutations, even a single nucleotide substitution (Orita et al., 1989; Cotton, 1993). Because of its sensitivity and technical ease, this method has been widely used to detect mutations causing various hereditary diseases in human (Ikonen et al., 1991; Dockhorn-Dworniczak et al., 1991; Baldwin et al., 1992), and was recently applied in plant breeding (Ikeda, 1994).

The purpose of this study is to clarify the phylogeny of *Ae. mutica* in the genera of *Triticum* and *Aegilops* by the PCR-SSCP analysis of the organellar DNAs. The phylogenetic study of *Triticum* and *Aegilops* species has been focused on the B genome donor of the polyploid wheats,

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especially on the Sitopsis section of *Aegilops*. The other *Aegilops* species have not been well analyzed. One of the interesting species is *Ae. mutica*. This species has many morphological characters which are shared with other genera in the tribe Triticeae (Ohta, 1991) and has high degrees of chromosome pairing in the F1 hybrids of many *Aegilops* species (Jones and Majisu, 1968; Ohta, 1991). These results suggest that *Ae. mutica* is an ancestral species in the genera *Triticum* and *Aegilops*. If this hypothesis is correct, we expect that *Ae. mutica* has a large intraspecific variation which may encompass the level of interspecific divergence of the other *Triticum* and *Aegilops* species. This served as the working hypothesis in this study. We report the results contrary to this hypothesis.

MATERIALS AND METHODS

Plant materials. Nine lines, each of *Ae. mutica* and *Ae. speltoides*, were used to investigate intraspecific variation. In addition, *T. aestivum* cv. Chinese Spring and eight alloplasmic lines of *T. aestivum* and *T. compactum* with different *Aegilops* cytoplasms were used as the reference

for the interspecific relationship. In total, 27 cytoplasms were analyzed (Table 1). Seeds of the euplasmic *Ae. mutica* and *Ae. speltoides* accessions were obtained from the Plant Germ-Plasm Institute, Kyoto University, Japan.

Organelar DNA regions investigated. Two regions of chloroplast and eight regions of mitochondrial genomes were investigated (Table 2). The chloroplast DNA regions include seven genes for the RubisCO large subunit (*rbcL*: Terachi et al., 1987; 3' flanking region of *rbcL*: Ogiwara et al., 1991), ATP synthase beta and epsilon subunits (*atpB* and *atpE*: Howe et al., 1985), the 47-kDa chlorophyll a-binding protein and the 10-kDa phosphoprotein of photosystem II, photosystem II polypeptide of 43 amino acids, apocytocrome b-563, and the 15-kDa polypeptide of the cytochrome b-f complex (*psbB*, *psbN*, *psbH*, and *petB*: Hird et al., 1991). The mitochondrial DNA regions include ten genes for the cytochrome oxidase subunit I, II and III (*coxI*: Bonen et al., 1987; *coxII*: Bonen et al., 1984; *coxIII*: Guarberto et al., 1990), the ATP synthase alpha subunit (*atpA*: Schulte et al., 1989), NADH dehydrogenase subunit I (*nad1-a*, *nad1-b*, *nad1-c* and *nad1-e*: Chap-

Table 1. Accessions used in this study

Euplasmic lines				
Species	Accession number		Section	Abbr.
<i>Aegilops mutica</i>	5501		Amblyopyrum	Mut1
"	5521		"	Mut2
"	5571		"	Mut3
"	5598		"	Mut4
"	5613		"	Mut5
"	5620		"	Mut6
"	5653		"	Mut7
<i>Aegilops speltoides</i>	2-5		Sitopsis	Spe1
"	5725B		"	Spe2
"	7712		"	Spe3
"	7776		"	Spe4
"	7786		"	Spe5
"	7802		"	Spe6
"	7822		"	Spe7
<i>Triticum aestivum</i>	C52		"	CS
Alloplasmic lines				
Nuclear donor	Cytoplasm donor	Code number	Section of cytoplasm donor	Abbr.
<i>Triticum compactum</i>	<i>Ae. caudata</i>	J02	Cylindropyrum	Cau
<i>Triticum aestivum</i>	<i>Ae. umbellulata</i>	F03	Polyides	Umb
"	<i>Ae. comosa</i>	C05	Comopyrum	Com
"	<i>Ae. uniaristata</i>	C07	"	Uni
"	<i>Ae. mutica</i>	C13	Amblyopyrum	Mut8
"	"	C14	"	Mut9
"	<i>Ae. speltoides</i>	C09	Sitopsis	Spe8
"	"	C17	"	Spe9
"	<i>Ae. sharonensis</i>	C10	"	Sha
"	<i>Ae. bicornis</i>	C12	"	Bic
"	<i>Ae. searsii</i>	C18	"	Sea
"	<i>Ae. longissima</i>	C20	"	Lon

Accession number of euplasmic lines is the KU number of Kyoto University (Tanaka, 1983). Code number of alloplasmic lines is that described by Tsunewaki and Tsujimoto (1983).

delaine and Bonen, 1991), maturase-like protein (*mat-r*: Chapdelaine and Bonen, 1991), and *orf25* (Bonen et al., 1990). PCR primers were designed based on the published sequences (Table 3). The amplified fragment in each region was shown in Figure 1.

PCR-SSCP analysis. The total DNA extraction method of Liu et al. (1990) was used for *Ae. mutica* accessions and all the alloplasmic lines. The CTAB method (Zimmer et al., 1981) was employed for the total DNA of *Ae. speltoides* accessions and *T. aestivum*. PCR amplification was car-

Table 2. The structural genes located in each region investigated

Region	Gene	Length (kb)	Length of coding sequence
Chloroplast DNA:			
<i>rbcL</i> region	<i>rbcL</i> , <i>atpB</i> , <i>atpE</i>	5.7	3.3
<i>psb</i> region	<i>psbB</i> , <i>psbN</i> , <i>psbH</i> , <i>petB</i>	4.0	2.3
total		9.7	5.6
Mitochondria DNA:			
<i>coxI</i> region	<i>coxI</i>	1.5	1.5
<i>coxII</i> region	<i>cox2</i>	1.9	0.7
<i>coxIII</i> region	<i>cox3</i>	0.7	0.7
<i>atp</i> region	<i>atpA</i>	1.7	1.5
<i>nada</i> region	<i>nad1-a</i>	1.1	0.4
<i>nadb</i> region	<i>nad1-b</i> , <i>nad1-c</i>	2.8	0.3
<i>nade</i> region	<i>nad1-e</i> , <i>met-r</i>	3.0	2.3
<i>orf25</i> region	<i>orf25</i>	0.4	0.4
total		13.1	7.8

The length of region is also shown.

Table 3. PCR primer sequences and restriction enzyme used to digest for each of investigated region

Region and subregion	Sense primer	Antisense primer	Enzyme
chloroplast DNA:			
<i>rbcL</i> region			
<i>atpB</i> and <i>atpE</i> subregion	5'CACAATAAGAGGGTCTACTC3'	5'ACCCAGAAGGAAACACCACT3'	<i>HinfI</i>
<i>rbcL</i> coding subregion	5'GAGTTATAGGGAGGGACTTATGTC3'	5'CGAATCTCGATTGTCAAGTCTC3'	<i>AluI</i>
3' flanking subregion	5'TTCGAGTTCGAGCCGGTAGATA3'	5'TGACAAGTTCCTCTTTACCC3'	<i>HinfI</i>
<i>psb</i> region			
<i>psbB</i> subregion	5'AGAAAGCAAGAATCCGCAGT3'	5'AGGATGGGAGATGTTTCCCA3'	<i>MspI</i>
<i>psbN</i> , <i>psbH</i> and <i>petB</i> subregion	5'GATTGGACATGCGAAACATC3'	5'TGATGCAGTCAAAACAGCCA3'	<i>DpnII</i>
mitochondrial DNA:			
<i>coxI</i> region	5'CTAACCACAAGGATATTGGGAC3'	5'AGTTCTCCAAAAGTATGAAAGGC3'	<i>MspI</i>
<i>coxII</i> region	5'TGATGCTGCGGAACCATGGCAA3'	5'GAGGATTAATTGATTGGATACCC3'	<i>HinfI</i>
<i>coxIII</i> region	5'ATGATTGAATTCTCAGAGGCATTTC3'	5'TGCGGCAAAGCCAACGTGATGC3'	<i>AluI</i>
<i>atp</i> region	5'CTGCGGAACCTCAGACTCTA3'	5'GGGTAGACTCTCAGCAGCTT3'	<i>DpnII</i>
<i>nada</i> region	5'GGTCCCATCCTTCAATATCA3'	5'TCTTCTATGTAGGGTATTCC3'	<i>DpnII</i>
<i>nadb</i> region	5'TCTTTCCAATGCCTCCTTCC3'	5'TTCCTTAGGCTCCGAGTCAC3'	<i>DpnII</i>
<i>nade</i> region	5'CGGCTCTTCGGAATGATTGG3'	5'GGGGCGGGGCATTTTCGGGG3'	<i>DpnII</i>
<i>orf25</i> region	5'TCTATTGTGCATCAAGTCCGAAG3'	5'TATATCGTCCTGAAGACGGATGCG3'	—

Fig. 1. The location and size of amplified fragment in investigated region for SSCP. (a), chloroplast DNA and (b), mitochondrial DNA. Broken line indicates the fragment amplified with the pair of primers shown in Table 3.

ried out with a DNA Thermal Cycler PJ2000 (Perkin-Elmer) programmed as follows: 6 min at 94°C (denaturing) followed by 30 cycles of 1 min at 94°C (denaturing), 2 min at 50°C (annealing) and 3 min at 72°C (extension) with a final extension of 7 min at 72°C. Reaction volume of the PCR amplification was 50 µl, containing 10 mM Tris-HCl

(pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 1.25 mM of each dNTP (Pharmacia), 0.3 mM of each primer, about 15 - 35 ng of the total DNA as template, 1.25 units of Taq DNA polymerase (AGS), and about 37 kBq (0.33 mM) of [α -³²P] dCTP (Amersham and ICN). The PCR product was digested with a restriction enzyme (Table 3) which cuts the

product into fragments suitable for SSCP analysis (less than 600 bp). After precipitation, the digested product was dissolved in 8 - 10 μ l of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and an equal volume of formamide loading buffer. The sample was heated at 95°C for 5 min and immediately placed on ice. Eight microliters of the sample was loaded onto non-denaturing 6% polyacrylamide gel (15 cm $\times$ 45 cm $\times$ 0.5 mm, 40:1 acrylamide/bisacrylamide and 10% glycerol) and subjected to electrophoresis in 1x TBE buffer (89 mM Tris-borate, pH8.0, 2 mM EDTA) at 2.8 - 4.0 W for 16 - 18 h at room temperature (usually less than 20°C). After electrophoresis, the gel was removed on chromatography paper (Whatman), dried at 80°C for 2 h and exposed to Fuji X-ray film at -20°C for 1 - 3 days using intensifying screens.

PCR-RFLP analysis. The *psb* region in chloroplast and *coxI* region in mitochondrial DNA were chosen for PCR-RFLP to determine the size of the fragments with SSCP. Because the migration distance of a fragment in non-denaturing acrylamide gel is usually not proportional to the size, it is impossible to determine the size of the variable fragments directly in SSCP gel. By comparing the banding patterns in SSCP and agarose gels, however, it is possible to assign a detected SSCP to a specific fragment predicted from the restriction enzyme digestion of a region. For the two regions which had SSCP, PCR amplification was repeated as conducted in the PCR-SSCP analysis without 32 P. After digesting the amplified fragment with the restriction enzyme used for PCR-SSCP, the digest was electrophoresed on 3% agarose gel (3 Nusieve : 1 Seakem agarose, FMC) in 1x TAE buffer with a size marker (100-bp Ladder, Gibco-BRL). By matching bands in two different gels, the fragments with SSCP were determined. From this information, the molecular basis of SSCPs was discussed.

RESULTS

PCR-SSCP analysis. In this study, two chloroplast and nine mitochondrial DNA regions in 27 accessions were investigated by PCR-SSCP analysis. A total of 268 bands were scored, of which 118 bands were found in chloroplast regions and 150 in mitochondrial regions. The average number of bands per kb was 11.1 for the total region investigated, and 10.8 and 11.4 for the chloroplast and mitochondrial regions, respectively. Of the 268 bands detected, 176 showed variation among accessions (65.7%). In the chloroplast, 82 out of 118 bands were variant among the accessions (69.5%), and in mitochondria 94 bands out of 150 bands were variant (62.7%). The two organellar genomes were not different from each other in the proportion of variable bands. Examples of SSCP autoradiograms are shown in Figure 2. Except for the *nada* region in mitochondria, at least one variant band was found in

the regions investigated. Among the regions, the 3' flanking region of the *rbcL* in chloroplast was the most variable. In this 1.4-kb region, a total of 30 bands were scored, and 25 were variant within and between species. This result is consistent with that of Ogihara et al. (1991), in which a high level of sequence variation was detected in the same region among three *Triticum* and *Aegilops* species.

Intra- and interspecific variations. The proportion of shared bands (PSB) and the distance defined as $D = 1 - \text{PSB}$ were calculated for each pair of accessions (Table 4). The intraspecific variation in terms of D of *Ae. mutica* ranged from 0.0034 to 0.0833, and that of *Ae. speltoides* from 0.0118 to 0.0943. It is noted that the average intraspecific distance of *Ae. mutica* (0.035) was lower than that of *Ae. speltoides* (0.053). This result is contrary to the expectation that *Ae. mutica* has a higher level of intraspecific variation if *Ae. mutica* is an ancestral species of the genera *Triticum* and *Aegilops* (Ohta, 1991). As for interspecific distance, the distances among species of the subsection Emarginata (*Ae. sharonensis*, *Ae. bicornis*, *Ae. searsii* and *Ae. longissima*) were smaller (0.031 - 0.073) than most of the other interspecific distances (0.065 - 0.282). All the Emarginata species had smaller distances with *Ae. mutica* than with *Ae. speltoides*. It can be noted that the distances between accessions of *Ae. speltoides* and *T. aestivum* (0.079 - 0.113) were smaller than those between any other species and *T. aestivum* (0.173 - 0.282). This result is consistent with the hypothesis that *Ae. speltoides* is the cytoplasmic donor (B genome donor) of the polyploid wheat (Tsunewaki et al., 1976; Ogihara and Tsunewaki, 1988; Dvorák and Zhang, 1990).

Phylogenetic tree. Based on the distances (D) calculated from the pooled data (both chloroplast and mitochondrial DNAs), two different phylogenetic trees were constructed by the unweighted pair-group method with arithmetic mean (UPGMA: Socal and Sneath, 1963, Fig. 3) and the neighbor joining method (NJ method: Saitou and Nei 1987, Fig. 4). On the whole, the two trees were similar. In both trees, *Ae. mutica*, *Ae. speltoides* (the subsection Truncata) and the other four species of the section Sitopsis (the subsection Emarginata) were separated into three different clusters. The *Ae. mutica* group is closer to the group of the Emarginata than to *Ae. speltoides*. The cluster of *Ae. speltoides* is distant from any other species. This topology is also contrary to the expectation that *Ae. mutica* is ancestral. It can be noted that *T. aestivum* was included in the cluster of *Ae. speltoides*. Phylogenetic trees (both UPGMA and NJ trees) were constructed based separately on the data of each chloroplast and mitochondrial DNA region (data not shown). Topologies of these two trees were essentially the same as those obtained from the pooled data. In both trees, *Ae. mutica*, *Ae. speltoides* and

the subsection Emarginata were separated into three different clusters.

PCR-RFLP analysis. Figures 5 and 6 show the comparison of the banding patterns of PCR-SSCP and PCR-

RFLP for the *psb* and *coxI* regions, respectively. In the SSCP gel of the *psb* region of the chloroplast, two variant bands were found (loss of the second band and gain of the fifth band in Fig. 5), which were specific to *Ae. umbellulata*. A comparison of banding patterns suggests

(a)

(b)

(c)

Fig. 2. Examples of autoradiograms showing SSCP in three regions. (a), *atpB* and *atpE* subregion of *rbcL* region; (b), 3' flanking region of *rbcL* region; (c), *coxI*: region. See Table 1 for the abbreviations for species: 1, Cau; 2, Umb; 3, Com; 4, Uni; 5, Spe8; 6, Sha; 7, Bic; 8, Mut8; 9, Mut9; 10, Spe9; 11, Sea; 12, Lon; 13, Mut1; 14, Mut2; 15, Mut3; 16, Mut4; 17, Mut5; 18, Mut6; 19, Mut7; 20, Spe1; 21, Spe3; 22, Spe4; 23, Spe5; 24, Spe6; 25, Spe7; and 26, CS.

that a new *DpnII* site (recognition sequence GATC) was gained in the 350-bp fragment. The new *DpnII* site probably produced an approximately 150-bp band and an approximately 200-bp band, indistinguishable from the 198-bp band. There are several "minus-one" sites, which would become the restriction site after one nucleotide mutation, in the 350-bp sequence of *T. aestivum* (Hird et al., 1991). Only one "minus-one" site (GGTC) in the *psbH* coding region could result in the two fragments of similar size detected in the *Ae. umbellulata* (155 and 195 bp). Although this change is nonsynonymous (Val to Ile), any other "minus-one" sites would not produce two fragments with a suitable size. These results suggest that the variable bands detected in the *psb* region were actually RFLP caused by a point mutation. Figure 6 shows the comparison of the *coxI* region in the mitochondrial DNA digested with *MspI*. Two variant bands were detected in the SSCP gel. *Ae. caudata* lost the third shortest band (173 bp), and *Ae. comosa* showed a size variation in the longest band (547 bp). In agarose gel, the 173-bp fragment still remained lost, but the size change in the 547-bp band was not seen. This result suggests that the size change in the 547-bp band was probably a true SSCP, however, the loss of the 173-bp band was not. An examination of the *coxI* sequence of *T. aestivum* (Bonen et al., 1987) indicates that the 173-bp band is located at the 3' end of the amplified region. This suggests that the *MspI* site in the 3' end of the band was lost in *Ae. caudata*. This change made the

Fig. 3. A phylogenetic tree constructed by UPGMA based on the SSCP of both chloroplast and mitochondrial DNA regions.

Table 4. Genetic distance between accessions based on the SSCP of both chloroplast DNA and mitochondrial DNA regions

	Cau	Umb	Com	Uni	Sha	Bic	Sea	Lon	Mut1	Mut2	Mut3	Mut4	Mut5	Mut6	Mut7	Mut8	Mut9	Spe1	Spe2	Spe3	Spe4	Spe5	Spe6	Spe7	Spe8	Spe9	CS
Cau	(108)																										
Umb	0.133	(86)																									
Com	0.187	0.118	(99)																								
Uni	0.089	0.146	0.152	(117)																							
Sha	0.130	0.139	0.134	0.124	(147)																						
Bic	0.180	0.139	0.107	0.121	0.033	(108)																					
Sea	0.168	0.186	0.177	0.178	0.058	0.073	(156)																				
Lon	0.152	0.161	0.165	0.165	0.031	0.064	0.042	(142)																			
Mut1	0.150	0.138	0.133	0.149	0.145	0.113	0.146	0.141	(154)																		
Mut2	0.147	0.136	0.131	0.145	0.138	0.115	0.145	0.138	0.020	(160)																	
Mut3	0.138	0.129	0.120	0.140	0.135	0.114	0.136	0.146	0.031	0.010	(160)																
Mut4	0.138	0.129	0.123	0.131	0.145	0.115	0.150	0.162	0.032	0.014	0.003	(147)															
Mut5	0.162	0.149	0.139	0.163	0.177	0.123	0.197	0.203	0.066	0.069	0.053	0.022	(157)														
Mut6	0.146	0.107	0.139	0.135	0.156	0.124	0.155	0.177	0.050	0.029	0.020	0.021	0.016	(124)													
Mut7	0.150	0.135	0.126	0.148	0.141	0.115	0.143	0.140	0.041	0.013	0.009	0.007	0.055	0.024	(160)												
Mut8	0.164	0.129	0.119	0.149	0.127	0.095	0.126	0.134	0.025	0.041	0.045	0.045	0.054	0.049	0.040	(165)											
Mut9	0.161	0.113	0.102	0.137	0.120	0.085	0.125	0.132	0.080	0.043	0.047	0.049	0.083	0.053	0.040	0.016	(141)										
Spe1	0.206	0.193	0.202	0.164	0.239	0.198	0.266	0.264	0.232	0.228	0.245	0.236	0.240	0.243	0.235	0.226	0.230	(142)									
Spe2	0.119	0.221	0.262	0.065	0.192	0.191	0.248	0.231	0.219	0.180	0.217	0.187	0.215	0.205	0.226	0.220	0.195	0.039	(52)								
Spe3	0.188	0.186	0.192	0.149	0.212	0.175	0.241	0.230	0.212	0.205	0.221	0.221	0.239	0.227	0.216	0.205	0.200	0.042	0.020	(151)							
Spe4	0.184	0.180	0.192	0.152	0.218	0.194	0.238	0.237	0.225	0.212	0.219	0.230	0.262	0.227	0.213	0.217	0.212	0.029	0.021	0.030	(153)						
Spe5	0.218	0.207	0.211	0.171	0.225	0.196	0.229	0.230	0.232	0.227	0.239	0.248	0.281	0.239	0.236	0.210	0.208	0.036	0.068	0.045	0.027	(150)					
Spe6	0.190	0.186	0.198	0.154	0.221	0.191	0.245	0.234	0.213	0.210	0.212	0.230	0.245	0.221	0.169	0.219	0.213	0.043	0.021	0.044	0.020	0.048	(152)				
Spe7	0.200	0.175	0.192	0.154	0.234	0.207	0.271	0.250	0.251	0.238	0.248	0.250	0.266	0.249	0.241	0.229	0.229	0.051	0.012	0.041	0.038	0.053	0.045	(137)			
Spe8	0.168	0.172	0.165	0.097	0.226	0.187	0.238	0.247	0.208	0.203	0.200	0.203	0.199	0.189	0.193	0.198	0.205	0.090	0.063	0.091	0.074	0.094	0.077	0.079	(139)		
Spe9	0.200	0.163	0.176	0.144	0.209	0.176	0.202	0.221	0.196	0.199	0.203	0.222	0.249	0.213	0.202	0.195	0.193	0.086	0.063	0.074	0.067	0.066	0.068	0.078	0.048	(134)	
CS	0.257	0.222	0.208	0.173	0.277	0.259	0.282	0.255	0.227	0.221	0.225	0.225	0.228	0.232	0.218	0.274	0.275	0.079	0.086	0.092	0.102	0.113	0.092	0.081	0.099	0.091	(76)

The numbers of scored bands are shown in parenthesis.

Fig. 4. A phylogenetic tree constructed by NJ method based on the SSCP of both chloroplast and mitochondrial DNA regions.

fragment 26 bp longer (the size of recognition sequence plus the size of the antisense primer) and indistinguishable from the 195-bp band both in SSCP and agarose gels. It can be concluded that one of variable bands in the *coxI* region was a RFLP. These results in *psb* and *coxI* regions suggest that the variations in this report were studied in terms of both SSCP and RFLP.

DISCUSSION

The results obtained in this study were contrary to the hypothesis based on the morphological and cytogenetical studies that *Ae. mutica* is an ancestral species in the genera of *Triticum* and *Aegilops* (Ohta, 1991). From this hypothesis *Ae. mutica* is expected to have a large intraspecific variation which might encompass the intraspecific variation of the other *Triticum* and *Aegilops* species. This hypothesis could also imply that the node of *Ae. mutica* could be one of the deepest in the phylogenetic tree of the genera. However, the intraspecific variation of *Ae. mutica* was smaller than that of *Ae. speltoides*. In the

phylogenetic tree, the cluster of *Ae. mutica* was nested in the section Sitopsis. Although these results suggest that *Ae. mutica* is not older than *Ae. speltoides*, it is possible to explain these findings from another point of view. It is known that the level of intraspecific variation depends on effective population size and mutation rate (Kimura, 1983). Because it is unlikely that the two species have different mutation rates in organellar DNAs, a possible explanation for the present result is that *Ae. speltoides* has a larger population size, i.e., a larger distribution area than *Ae. mutica*. Actually, *Ae. speltoides* is found in the Middle East, the entire Fertile Crescent arc, while *Ae. mutica* is distributed only in Turkey and Armenia (Eig,

Fig. 5. Comparison of PCR-RFLP and PCR-SSCP of the *psbN* and *psbH* regions in chloroplast. (a), a diagram of PCR-RFLP banding pattern in agarose gel stained with ethidium bromide. (b), a diagram of PCR-SSCP banding pattern in autoradiogram. See Table 1 for the abbreviation for accessions. 'M' indicates the 100-bp ladder size marker.

Fig. 6. Comparison of PCR-RFLP and PCR-SSCP of the *coxI* region in mitochondria. (a), PCR-RFLP banding pattern in agarose gel stained with ethidium bromide. (b), PCR-SSCP banding pattern in autoradiogram. See Table 1 for the abbreviation for accessions. 'M' indicates the 100-bp ladder size marker.

1929; Slageren, 1994). Because the number of species and accessions is too small to conclude the present result, both intra- and interspecific variations of other diploid *Triticum* and *Aegilops* species must be studied in the future.

It was shown that the species in the section *Sitopsis* were separated into two distinct groups in the phylogenetic trees. One group contained *Ae. speltooides* (the subsection *Truncata*), and the other contained four species of the subsection *Emarginata*. This topology has been obtained by RFLP analyses of organellar and nuclear DNAs (Ogihara and Tsunewaki, 1988; Murai et al., 1989; Terachi et al., 1990; Terachi and Tsunewaki, 1992; Sasanuma et al., 1996). These results suggest that the section *Sitopsis* consists of two subsections phylogenetically distant from

each other and that *Ae. speltooides* is of old origin in the genus. A relatively high level of polymorphism in *Ae. speltooides* reported in this study and by Sasanuma et al. (1996) may suggest the antiquity of *Ae. speltooides* in the genus. In addition, it was shown that *T. aestivum* was included in the cluster of *Ae. speltooides*. This result suggests that *Ae. speltooides* is the cytoplasmic donor (B genome donor) of common wheat as the previous studies have shown (Tsunewaki et al., 1976; Ogihara and Tsunewaki, 1988; Dvorák and Zhang, 1990; Terachi and Tsunewaki, 1992).

It has been reported that the nucleotide substitution rate of mitochondrial DNA is lower than that of chloroplast DNA (Holwerda et al., 1986; Wolfe et al., 1987; Terachi and Tsunewaki, 1992). In this study it was ex-

pected that the mitochondrial DNA would have fewer variable bands than the chloroplast, if the SSCP analysis employed reveals only nucleotide variation. However, there was only a small difference in the proportion of variable bands between chloroplast (69.5%) and mitochondrial (62.7%) DNAs. One of the explanations is that there was a difference in the proportion of coding sequence between chloroplast and mitochondrial DNA regions investigated. The results show that 57.8% of the chloroplast regions was coding sequence, while 59.4% was in the mitochondrial regions. A similar proportion of coding sequence in the mitochondrial regions is not consistent with this explanation. Another explanation is that the PCR-SSCP analysis detected structural rearrangements as well as nucleotide substitutions in mitochondrial DNA. It is known that the mitochondrial genome in plants undergoes intramolecular recombinations to generate structural variations (Fauron et al., 1995). This might have inflated the level of variation in mitochondrial DNA. The exact reason is not yet clear.

It has been shown that SSCP has a sensitivity to detect at least 80% of all the DNA variation in a given fragment (Cotton, 1993). In this study, we actually combined SSCP analysis with RFLP analysis. The PCR-RFLP analysis indicated that the variations detected in this study were the mixture of RFLP and SSCP. We might have detected variations in organellar DNAs at a better efficiency than achieved by each of the techniques separately. Variations were detected in most of the regions investigated, and the phylogenetic relationship revealed in this study was consistent with those obtained by the previous studies. Considering these results, it can be concluded that PCR-SSCP analysis is useful to investigate intra- and interspecific variations in DNA sequences of *Triticum* and *Aegilops*, and the other plant species as well.

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