Molecular Variation in Chloroplast DNA Regions in Ancestral Species of Wheat

Naohiko T. Miyashita,* Naoki Mori† and Koichiro Tsunewaki*

**Laboratory of Genetics, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan, and †Laboratory of Genetics, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657, Japan*

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ABSTRACT

Restriction map variation in two 5–6-kb chloroplast DNA regions of five diploid *Aegilops* species in the section Sitopsis and two wild tetraploid wheats, *Triticum dicoccoides* and *Triticum araraticum*, was investigated with a battery of four-cutter restriction enzymes. A single accession each of *Triticum durum*, *Triticum timopheevi* and *Triticum aestivum* was included as a reference. More than 250 restriction sites were scored, of which only seven sites were found polymorphic in *Aegilops speltoides*. No restriction site polymorphisms were detected in all of the other diploid and tetraploid species. In addition, six insertion/deletion polymorphisms were detected, but they were mostly unique or species-specific. Estimated nucleotide diversity was 0.0006 for *A. speltoides*, and 0.0000 for all the other investigated species. In *A. speltoides*, none of Tajima's *D* values was significant, indicating no clear deviation from the neutrality of molecular polymorphisms. Significant non-random association was detected for three combinations out of 10 possible pairs between polymorphic restriction sites in *A. speltoides*. Phylogenetic relationship among all the plastotypes (plastid genotype) suggested the diphyletic origin of *T. dicoccoides* and *T. araraticum*. A plastotype of one *A. speltoides* accession was identical to the major type of *T. araraticum* (*T. timopheevi* inclusively). Three of the plastotypes found in the Sitopsis species are very similar, but not identical, to that of *T. dicoccoides*, *T. durum* and *T. aestivum*.

MOLECULAR population genetic study, which has been done mainly by using animals, has contributed greatly to our understanding of genetic diversity at the DNA level and underlying genetic mechanisms (KIMURA 1983; GILLESPIE 1991). But plant species have not been investigated as much because of experimental difficulties associated with long life span, large individual size, and labor-taking artificial crossing. However, recent progress in plant molecular biology has allowed study of plant species from the viewpoint of population and evolutionary genetics at the most fundamental level of DNA. To obtain a general picture of organic evolution, the information from plant species is needed, since the information of intra- and interspecific variation only from animals might have biased our knowledge of the nature of genetic variation.

Polyploidy evolution of *Triticum* (wheat) and *Aegilops* species is one of the most investigated subjects in plant genetics. *Triticum* species are classified into four species complexes according to the polyploidy and genome constitution: Einkorn (AA), Emmer (AABB), Timopheevi (AAGG) and common wheat (AABBDD), while *Aegilops* species are divided into six sections and their genome constitutions are more complicated than *Triticum* [see LILIENFELD (1951) for review]. It is well known that hexaploid common wheat (*Triticum aestivum*) has originated from the alloploidy between Emmer wheat and *Aegilops squarrosa* (DD) (KIHARA 1944; MCFADDEN and SEARS 1946). Molecular studies on chloroplast and mitochondrial DNA of *Triticum* and *Aegilops* species have shown that *T. aestivum* and Emmer wheats share the identical or very similar restriction fragment length polymorphism (RFLP) and sequence variations. These results have given evidence that Emmer, cultivated or wild, wheat had served as the mother of *T. aestivum* (TSUNEWAKI and OGIHARA 1983; BOWMAN *et al.* 1983; GRAUER *et al.* 1989; TERACHI and TSUNEWAKI 1992). Emmer wheat is also the product of alloploidy between two diploid species (LILIENFELD 1951). Although the A genome donor of Emmer wheat (the grandfather of common wheat) has been determined to be Einkorn wheat by cytological and molecular studies, probably *Triticum urartu* (CHAPMAN *et al.* 1976; DVORAK *et al.* 1988; TAKUMI *et al.* 1993), there is still controversy about the donor of the B genome (the grandmother of common wheat). The most likely candidate species of the B genome donor is thought to belong to the Sitopsis section of *Aegilops* (*Aegilops speltoides*: SARKAR and STEBBINS 1956; RILEY *et al.* 1958; OGIHARA and TSUNEWAKI 1988; DVORAK and ZHANG 1990; *Aegilops sharonensis*: KUSHNIR and HALLORAN 1981, *Aegilops longissima*: VITTOZI and SILANO 1976; GERLACH *et al.* 1978, *Aegilops searsii*: FELDMAN 1978, *Aegilops bicornis*: SEARS 1956), of which *A. speltoides* is most favored from the RFLP data of both chloroplast and nuclear DNA (OGIHARA and TSUNEWAKI 1988; DVORAK and ZHANG 1990). However, conclusive evidence is still awaited to resolve the last remaining questions in wheat evolution.

Despite these extensive studies on wheat phylogeny, only very few accessions from each species have been

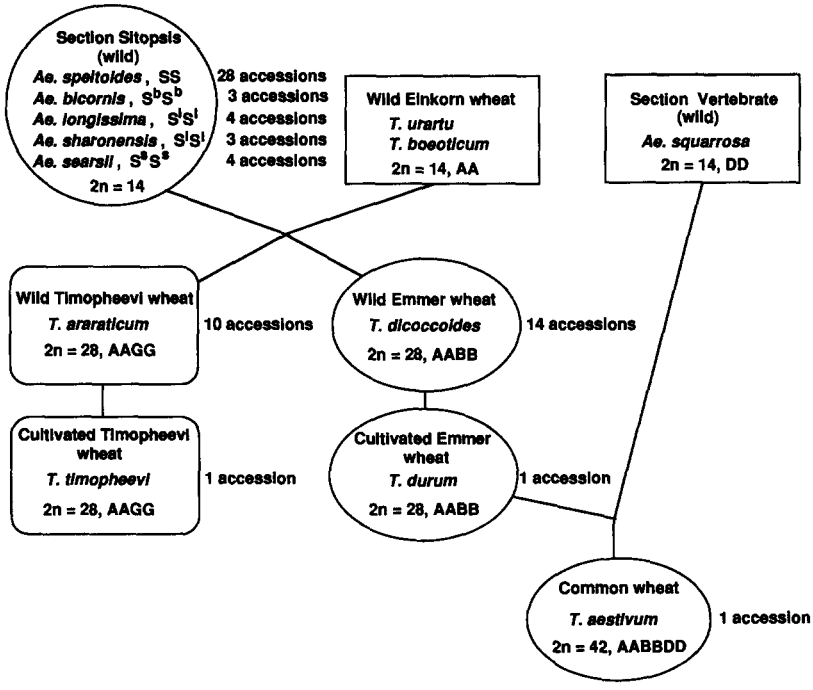

FIGURE 1.—*Triticum* and *Aegilops* species investigated. Their phylogenetic relationship and the number of accessions are also shown.

investigated. Intraspecific variation of *Triticum* and *Aegilops* species has not been considered extensively. The importance of knowing the intraspecific variation in order to study the phylogenetic relationship between species has been well recognized, since a gene tree based on a particular gene or region analyzed might be different from the species tree (TAJIMA 1983; TAKAHATA and NEI 1985; NEI 1987), and it is possible to infer genetic mechanisms operating in the evolutionary process by contrasting the interspecies divergence and intraspecific polymorphism (MCDONALD and KREITMAN 1991; OHTA 1993). In this report, by using five diploid *Aegilops* and two tetraploid wheat species, which might have been involved in the polyploidy evolution toward common wheat, restriction map variation of two chloroplast DNA regions was investigated. There are two purposes. First the aim is made to estimate the amount of intraspecific variation at the DNA level in those species, as the first step for wheat population genetics. Second, since the phylogenetic relationship between those species is well established, it is expected to reveal some new aspects associated with the maternal lineage in polyploidy evolution by studying the intraspecific variation in chloroplast DNA regions of these ancestral species of common wheat.

MATERIALS AND METHODS

Plant materials: Figure 1 shows the number of accessions of *Aegilops* and *Triticum* species used in this study. The Sitopsis section is divided into two subsections, Emarginata and Truncata. *A. speltoides* belongs to the latter and *Aegilops bicornis*, *Aegilops longissima*, *Aegilops sharonensis* and *Aegilops searsii* are placed in the former subsection. This figure also shows the nuclear genome constitution of each species and summarizes the possible phylogenetic relationship among these species based on previous results. Accessions originated from different localities were used to estimate the level of variation at the species level. They were sampled in Iran, Iraq, Turkey and Israel, which cover the main distribution area of all the species studied in this report. In addition, 14 accessions from Maras (Turkey) population of *A. speltoides* were studied. In the above investigation an accession from this population was revealed to have a plastotype (s6 in Figure 3) which is very similar to that of Emmer and common wheat. Using the data obtained, the intraspecific variation in a single habitat was estimated. One accession each of *Triticum durum*, *Triticum timopheevi* (both cultivated tetraploid) and *Triticum aestivum* (cultivated hexaploid) were included as a reference.

All the accessions used have been maintained by continuous selfing in the Plant Germ-Plasm Institute, Kyoto University. Accession numbers and passport data are available from the first author upon request. All of the seeds were supplied by the Plant Germ-Plasm Institute, Kyoto University.

Restriction map analysis: Total DNA extraction method follows LIU *et al.* (1990). Restriction map variation of chloroplast DNA was investigated by the "four-cutter analysis" of KREITMAN and AGUADÉ (1986). Both of restriction site and length changes cause the variation in restriction map. Procedures for digestion, gel electrophoresis, electroblotting and hybridization follows MIYASHITA (1990). A total of 13 four-cutter restriction enzymes were used. Two regions of chloroplast DNA shown in Figure 2 were subjected to the present analyses. For each region, three probes for hybridization were prepared by polymerase chain reaction (PCR) amplification. Primers for PCR were designed based on the published sequences of each region (HOWE *et al.* 1985; TERACHI *et al.* 1987; HIRD *et. al.* 1991; OGIHARA *et al.* 1991). Those primer sequences are shown in Table 1.

RESULTS AND DISCUSSION

Restriction map variation: Figure 2 shows the locations of polymorphisms in the regions studied, and Table 2 summarizes the frequency of detected molecular variations. Overall, more than 250 restriction sites

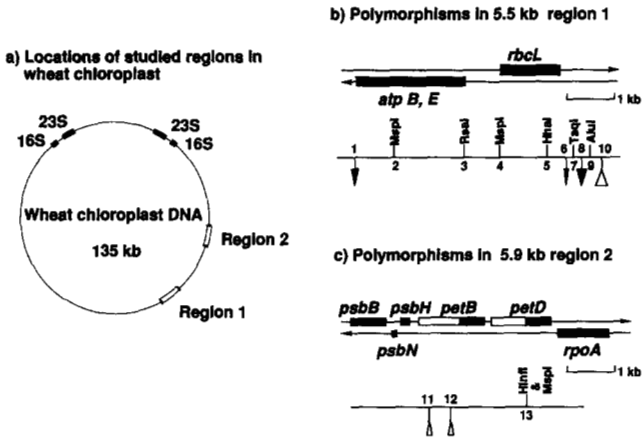

FIGURE 2.—(a) Chloroplast DNA regions investigated in this report. Polymorphisms detected in region 1 is shown in (b) and in region 2 in (c). Solid bars indicate transcribed region, and open bar intron. Arrow indicates the direction of transcription. Detected polymorphisms (both site and length mutation) are numbered sequentially.

were scored. Seven restriction site polymorphisms were detected only in *A. speltoides*. No restriction site polymorphisms were found in the other diploid *Aegilops* species nor in the tetraploid *Triticum* species (*Triticum dicoccoides* and *Triticum araraticum*). Estimates of nucleotide variation are shown in Table 3. It is clear that the level of nucleotide variation is very low in the chloroplast genome. None of TAJIMA's (1989) *D* test values was significant. This result suggests that the distribution of molecular polymorphisms (restriction site and insertion/deletion) does not deviate from the neutrality expectation, although the level of polymorphism may be too low for this test to be significant. Region 2 has a lower variation than region 1. This difference may be related to the higher density of transcriptional units in region 2. As noted by OGIHARA *et al.* (1988, 1991), the 3′ region of *rbcL* seems to be more variable than the other regions. The pooled estimates of nucleotide variation ($\theta = 0.85 \times 10^{-3}$ and $\pi = 0.60 \times 10^{-3}$) are about one order lower than those obtained in Drosophila nuclear DNA regions (AQUADRO 1992). However, the chloroplast genome is maternally inherited and is comparable to the haploid genome. The nucleotide variation in the chloroplast genome could be one-fourth of that of the diploid nuclear genome, if nucleotide mutation rates in the Drosophila nuclear genome and *A. speltoides* chloroplast genome are the same. Adjusting for this factor, it is noted that the level of nucleotide variation in the chloroplast genome of *A. speltoides* is the same order of magnitude as that of the Drosophila nuclear genome. This result may suggest that the maternal effective population size of *A. speltoides* is the same order as that of Drosophila. This seems unlikely, because *Drosophila* species (excluding some endemic species) generally have a much larger distribution area and census number than *A. speltoides* whose distribution area is restricted only to the Middle East. At the moment, it is not meaningful to compare the levels of nucleotide variation between these two species, before studying the variation in other genes in other genomes of this grass genus. The pooled estimates of nucleotide variation for the Maras population of *A. speltoides* were $\theta = 0.30 \times 10^{-3}$ and $\pi = 0.26 \times 10^{-3}$ which are the same order of magnitude as the species level, although the values are a little smaller. In this Maras population, only two polymorphic restriction sites were detected among 14 accessions.

Insertion/deletion polymorphisms were detected specifically in some particular species in a very low frequency. These species-specific insertion/deletion polymorphisms cause the variation of plastotypes of tetraploid species, in which no restriction site polymorphisms were detected. These results may suggest that maintenance mechanism and mutation process for insertion/deletion are different from those of restriction site polymorphism, as noted in Drosophila (AQUADRO *et al.* 1986; GOLDING *et al.* 1986; IIZUKA 1989).

In the other plant species, the intraspecific variation of chloroplast DNA was investigated with six-cutter restriction enzymes (pearl millet: CLEGG *et al.* 1984b; barley: CLEGG *et al.* 1984a; lupine: BANKS and BIRKY 1985; white pine: WHITE 1990). These studies have also shown that the level of variation in the chloroplast genome was very low. The result obtained in this study is consistent with those previous reports.

Table 2 shows detected plastotypes and their frequencies. *A. speltoides* has nine plastotypes, but the major plastotype, s3, is predominant at the species level and also in a single location (Maras). Plastotype (haplotype) diversity is 0.72 at the species level, and 0.37 in the Maras population (NEI and TAJIMA 1981). Lower plastotype diversity in a single location may suggest a limited migration among subpopulations. The other four Sitopsis species, all of the subsection Emarginata, share only two plastotypes, which are not found in *A. speltoides*. It could be noted that the *Rsa*I site in *atpE* gene (polymorphism #3) delineates Emmer and Timopheevi lineages, although these two complexes have very similar plastotypes. This *Rsa*I site is detected in all of the *Aegilops* species and found polymorphic only in *A. speltoides* from Maras. Two plastotypes s6 and sb1 are very similar to d11 of *Triticum dicoccoides*, *Triticum durum* and *Triticum aestivum*, except for the presence of two unique insertions (#10 and #12), and one insertion (#1) and one restriction site (#9), respectively. The plastotype m11 which was found in the Maras population is identical to that of the major type of *Triticum araraticum* and *Triticum timopheevi*, and differs from the plastotype of Emmer and common wheats only by the *Rsa*I site (#3).

TABLE 1

PCR primers used to amplify two regions of chloroplast DNA for hybridization probes

	Sense primer	Antisense primer
Region 1:		
atpB and *atpE* subregion (probe 1, 2.8 kb)	5′ CAC AAT AAG AGG GTC TAC TC 3′	5′ TCC CAG AAG GAA ACA CCA CT 3′
rbcL coding subregion (probe 2, 1.7 kb)	5′ GAG TTA TAG GGA GGG ACT TAT GTC 3′	5′ CGA ATC TCG ATT TGT CAA GTC TC 3′
3′ flanking subregion of *rbcL* (probe 3, 1.4 kb)	5′ TTC GAG TTC GAG CCG GTA GAT A 3′	5′ GTT CTA CCC ATA TGT GTT CTG A 3′
Region 2:		
psbB subregion (probe 4, 2.1 kb)	5′ AGA AAG CAA GAA TCC GCA GT 3′	5′ AGG ATG GGA GAT GTT TCC CA 3′
psbN, *psbH* and *petB* subregion (probe 5, 2.0 kb)	5′ GAT TTG ACA TGC GAA ACA TC 3′	5′ TGA TGC AGT CAA AAC AGC CA 3′
petB, *petD* and *rpoA* subregion (probe 6, 2.0 kb)	5′ ATG ATC CTG CAT GTA TTT CG 3′	5′ ACT CTA GAG AAG CAT CTC CC 3′

TABLE 2

Summary of molecular variations detected in two chloroplast DNA regions in *Triticum* and *Aegilops* species

Species	Plastotype	No. of accessions	Polymorphism 1	2	3	4	5	6	7	8	9	10	11	12	13
A. speltoides	s1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
A. speltoides	s2	2	0	0	0	1	0	0	1	0	0	0	0	0	0
A. speltoides	s3	14 (11)[a]	0	0	0	0	0	0	1	0	0	0	0	0	0
A. speltoides	s6	1 (2)	0	0	1	0	1	0	1	0	0	1	0	1	0
A. speltoides	s7	3	0	0	0	0	1	0	1	0	1	0	0	0	0
A. speltoides	s11	4	0	1	0	1	0	0	1	0	0	0	0	0	0
A. speltoides	s12	2	0	0	0	1	0	0	1	0	0	0	0	0	1
A. speltoides	s16	1	0	1	0	1	0	0	1	0	0	0	0	0	1
A. speltoides	m11	(1)	0	0	0	0	1	0	1	0	0	0	0	0	0
A. longissima	ls1	4	1	0	1	0	1	0	1	1	1	0	0	0	0
A. sharonensis	ls1	2	1	0	1	0	1	0	1	1	1	0	0	0	0
A. sharonensis	sb1	1	1	0	1	0	1	0	1	0	1	0	0	0	0
A. bicornis	sb1	3	1	0	1	0	1	0	1	0	1	0	0	0	0
A. searsii	sb1	4	1	0	1	0	1	0	1	0	1	0	0	0	0
T. araraticum	m11	8	0	0	0	0	1	0	1	0	0	0	0	0	0
T. araraticum	a4	2	0	0	0	0	1	0	1	0	0	0	1	0	0
T. dicoccoides	d11	12	0	0	1	0	1	0	1	0	0	0	0	0	0
T. dicoccoides	d14	1	1	0	1	0	1	0	1	0	0	0	0	0	0
T. dicoccoides	d17	1	0	0	1	0	1	1	1	0	0	0	0	0	0
T. timopheevi	m11	1	0	0	0	0	1	0	1	0	0	0	0	0	0
T. durum	d11	1	0	0	1	0	1	0	1	0	0	0	0	0	0
T. aestivum	d11	1	0	0	1	0	1	0	1	0	0	0	0	0	0

Numbers of polymorphisms correspond to those in Figure 2; 1 indicates the presence, and 0 absence.
[a] The number in parentheses is for the Maras population study.

Non-random association between molecular polymorphisms: Because of no recombination in chloroplast genome, it is expected that non-random association will be generated through mutation and random genetic drift without assuming natural selection (HILL and ROBERTSON 1968; OHTA and KIMURA 1969). It is of interest to investigate the level of non-random association in chloroplast genome from a view point of population genetics. Non-random association between molecular polymorphisms was tested only for *A. speltoides* (Table 4). Polymorphisms detected more than once in the sample were used for the analysis. Three out of 10 possible combinations show a significant departure from random association. It can be noted that between the *Msp*I site in *atpB* (#2) and *Hin*fI in *petD* (#13) there are all four possible allelic combinations. This observation may imply that either recombination or recurrent mutation has occurred in chloroplast genome.

Molecular phylogeny: Figure 3 is the summary network of the plastotypes detected in this study based on the neighbor-joining method (SAITOU and NEI 1987). One hypothetical plastotype in the diploid species was necessary in the network in order to accommodate all of the diploid species in one group. Otherwise, the plastotype d11 of *Triticum dicoccoides* is placed in the middle of diploid network, and three plastotypes (s6, sb1 and ls1) would be separated from the rest of *A. speltoides*. It seems very unlikely to assume that the tetraploid species was involved in the differentiation process of the diploid species. A similar relationship is also ob-

TABLE 3

Estimates of intraspecific nucleotide variation in chloroplast genome

Region and taxon[a]	No. of accessions	No. of sites scored	No. of polymorphisms		θ^b (10^{-3})	π^c (10^{-3})	*Tajima's D*[d]	
			Site	Length			Site variation	Length variation
Region 1								
A. speltoides	28	182	6	1	1.26	0.83	−0.46 NS	−1.16 NS
Emarginata	14	155	0	1	0	0	NA	NA
T. dicoccoides	14	150	0	2	0	0	NA	NA
T. araraticum	10	150	0	0	0	0	NA	NA
Region 2								
A. speltoides	28	130	1	1	0.29	0.18	−0.41 NS	−1.16 NS
Emarginata	14	111	0	0	0	0	NA	NA
T. dicoccoides	14	109	0	0	0	0	NA	NA
T. araraticum	10	109	0	1	0	0	NA	NA
Pooled								
A. speltoides	28	312	7	2	0.85	0.60	−0.52 NS	−1.53 NS
Emarginata	14	266	0	1	0	0	NA	NA
T. dicoccoides	14	259	0	2	0	0	NA	NA
T. araraticum	10	259	0	1	0	0	NA	NA

[a] Emarginata is a subsection of Sitopsis, a section of *Aegilops*, that includes four species, *A. bicornis*, *A. longissima*, *A. sharonensis* and *A. searsii*.
[b] θ, estimate of Nu (HUDSON 1982).
[c] π, nucleotide diversity (NEI and LI 1979).
[d] NS, nonsignificant; NA, not applied.

TABLE 4

Non-random association between polymorphic restriction sites in *A. speltoides* chloroplast genome

Polymorphic sites compared	Allelic combination				χ^2	Significance
	0-0	0-1	1-0	1-1		
*Msp*I (*atpB*)/*Msp*I (*rbcL* 5′)	18	3	0	5	13.93	$P < 0.01$
*Msp*I (*atpB*)/*Hha*I (*rbcL*)	19	4	5	0	1.01	NS[a]
*Msp*I (*atpB*)/*Alu*I (*rbcL* 3′)	20	3	5	0	0.73	NS
*Msp*I (*atpB*)/*Hin*fI (*petD*)	21	2	4	1	0.55	NS
*Msp*I (*rbcL* 5′)/*Hha*I (*rbcL*)	14	4	8	0	2.10	NS
*Msp*I (*rbcL* 5′)/*Alu*I (*rbcL* 3′)	15	3	8	0	1.51	NS
*Msp*I (*rbcL* 5′)/*Hin*fI (*petD*)	18	0	5	3	7.63	$P < 0.05$
*Hha*I (*rbcL*)/*Alu*I (*rbcL* 3′)	24	0	1	3	20.16	$P < 0.001$
*Hha*I (*rbcL*)/*Hin*fI (*petD*)	21	3	4	0	0.56	NS
*Alu*I (*rbcL* 3′)/*Hin*fI (*petD*)	22	3	3	0	0.40	NS

[a] NS, nonsignificant ($P > 0.05$).

tained by the maximum parsimony method (data not shown). The species which has this hypothetical plastotype would be the B genome donor of Emmer and common wheat. The plastotypes s6 (*A. speltoides*) and sb1 (*A. bicornis*, *A. sharonensis* and *A. searsii*) are very similar to d11 of the major type of *T. dicoccoides* (*T. durum* and *T. aestivum*, inclusively). From the tree, it can be noted that if the diploid species other than *A. speltoides* occupied at the position of the hypothetical plastotype, the network of *A. speltoides* is disrupted. Therefore, *A. speltoides* seems to be the species which has the hypothetical plastotype of the possible B genome donor. To confirm this point, different regions are currently under investigation.

There are four points to be stressed from the network. First, as mentioned above, the section Sitopsis contains species whose plastotypes are very similar to that of *T. aestivum*, *T. durum* and the major plastotype of *T. dicoccoides*. This result is consistent with the present consensus that the B genome donor of common wheat belongs to this section. Second, the tree suggests a diphyletic origin of Emmer and Timopheevi wheats. Here, diphyletic origin means that these two tetraploid species appeared from two different combinations of parental species (or individuals) at two different times. On the other hand, monophyletic origin implies that one of the tetraploids was the ancestor of the other, indicating that only one pair of diploid species (or individual), and consequently one maternal diploid species, was involved in the formation of these tetraploid species. It has been shown that *T. dicoccoides* has much larger RFLP variation at the nuclear DNA level than *Triticum araraticum*, suggesting that *T. dicoccoides* is older than *T. araraticum* (MORI 1991; TSUNEWAKI *et al.* 1993). Thus, if a monophyletic origin is assumed, *T. araraticum* must have appeared from *T. dicoccoides*. However, this scheme is unlikely. In order for this scheme to happen, the *Rsa*I site in *atpE* (#3) in par-

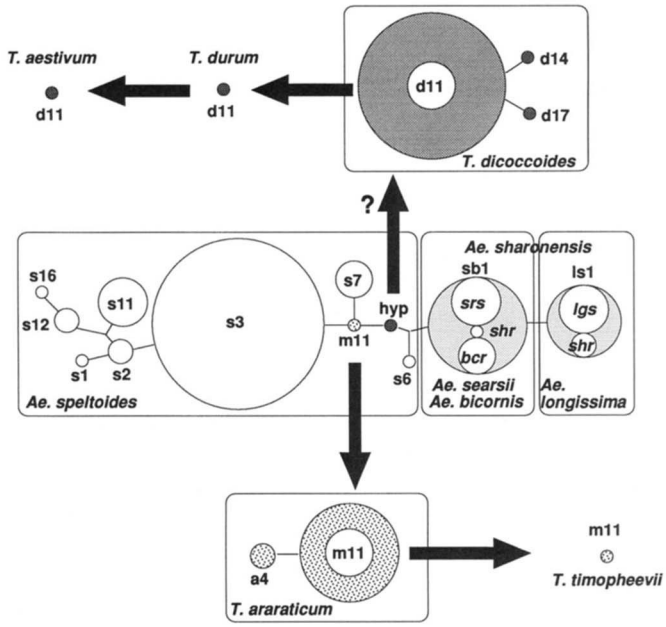

FIGURE 3.—Summary network of plastotypes detected in this study, based on the neighbor-joining method. Diameter of circle is proportional to the number of accessions having respective plastotype. Thick arrow indicates the direction of evolution. Plastotypes are shown in Table 2. "hyp" means hypothetical.

ticular which must have been gained in the B genome donor was transmitted to *T. dicoccoides* and again lost at the origination of *T. araraticum*. If this *Rsa*I site has a higher mutation rate, it would be expected that this site could be more polymorphic. But this is not the case in any species in the Sitopsis section. This study showed that one accession of *A. speltoides* has the identical plastotype of the major type of wild *T. araraticum* and the cultivated *Triticum timopheevi*. This result contrasts with the failure of finding Emmer wheat plastotype in the diploid species investigated here, and suggests that the appearance of Timopheevi wheat is more recent than that of Emmer wheat. These two tetraploid wheats may have appeared at the two different times and been originated from different diploid mothers with a very close genetic relationship, as can be seen from the network. It is curious to note that the two plastotypes s6 and m11 of *A. speltoides* which are very close to those of the tetraploid species were both found in the accessions sampled in Maras, Turkey. At this moment it is not clear whether this is just a coincidence or the area near Maras of Turkey was the birthplace of these tetraploids. Third, no restriction site polymorphisms were detected in the two tetraploid species, and only very rare insertion/deletion variations were found. The observation agrees with the idea that the tetraploids came after diploid species, and suggests that very few maternal ancestral individuals (maybe only one) were involved in the formation of each of these tetraploid species. Finally, the frequency of plastotypes indicates that new species (tetraploid) do not necessarily come from the major type of original species (diploid) as the mother, if the frequency of different plastotypes did not change so greatly since the appearance of the new species. As can be seen, plastotypes of both tetraploids are not related to the major plastotype of diploid species.

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