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Evaluation of allelic diversity at chloroplast microsatellite loci among common wheat and its ancestral species

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Abstract Twenty four chloroplast microsatellite loci having more than ten mononucleotide repeats were identified from the entire chloroplast DNA sequence of common wheat, *Triticum aestivum* cv Chinese Spring. For each microsatellite, a pair of primers were designed to produce specific PCR products in the range of 100–200 bp. The allelic diversity at the microsatellite loci was evaluated using 43 accessions from 11 *Triticum* and *Aegilops* species involved in wheat polyploid evolution. Polymorphic banding patterns were obtained at 21 out of 24 chloroplast microsatellite loci. The three monomorphic microsatellites were found to be located in coding regions. For the polymorphic microsatellites, the number of alleles per microsatellite ranged from 2 to 7 with an average of 4.33, and the diversity values (H) ranged from 0.05 to 0.72 with an average of 0.47. Significant correlations ($P<0.01$) were observed between the number of repeats and the number of alleles, and between the number of repeats and diversity value, respectively. The genetic diversity explained by chloroplast microsatellites and nuclear RFLP markers were compared using 22 tetraploid accessions. Although the number of alleles for nuclear RFLP markers was found to be higher than that for chloroplast microsatellites, similar diversity values were observed for both types of markers. Among common wheat and its ancestral species, the percentages of common chloroplast microsatellite alleles were calculated to examine their phylogenetic relationships. As a result, Timopheevi wheat species were clearly distinguished from other species, and Emmer and common wheat species were divided into two main groups, each consisting of a series of wild and cultivated species from tetraploid to hexaploid. This indicates that the two types of chloroplast genomes of common wheat might have independently originated from the corresponding types of wild and cultivated Emmer wheat species.

Keywords Chloroplast microsatellites · Simple sequence length polymorphism (SSLP) · Allelic diversity · Wheat species · Polyploid evolution

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Introduction

Simple sequence repeats (SSRs), or microsatellites, are abundant and well-distributed throughout the nuclear genomes of eukaryotes. Simple sequence length polymorphism (SSLP), caused by variation in the number of repeat units, can be easily detected by PCR using pairs of primers designed from unique sequences bordering the SSR motifs. Because of the high level of polymorphism and the genome-wide distribution, microsatellites have become useful markers for genome analysis. Highly saturated microsatellite maps were first developed for mammals, such as human (Dib et al. 1996) and mouse (Dietrich et al. 1996). For plant species, microsatellite markers have been placed on the molecular framework maps mainly composed of RFLP markers in several crop species, including rice (Chen et al. 1997; Temnykh et al. 2000), maize (Senior et al. 1996), wheat (Röder et al. 1998), barley (Liu et al. 1996) and soybean (Cregan et al. 1999).

SSRs were also identified in the plant chloroplast genomes. Powell et al. (1995b) made a large-scale survey of SSRs through the computer searches of the GenBank sequence database. They found 237 microsatellites with more than ten repeats in the complete chloroplast sequences of six plant species. Since the size of the chloroplast genome is much smaller than that of the nuclear

Table 1 Plant materials used for chloroplast microsatellite analysis

Species	Genome	Code	Accession	Origin
T. monococcum	AA	Mnc-1	KU3640	Turkey
Ae. squarrosa	DD	Sqr-1	KU2080	Iran
Ae. speltoides	SS	Spt-1	KU2284	Turkey
T. araraticum	AAGG	Ara-1	KU1927	Armenia
T. araraticum	AAGG	Ara-2	KU1943	Turkey
T. araraticum	AAGG	Ara-3	KU8912	Turkey
T. araraticum	AAGG	Ara-4	KU8940	Turkey
T. araraticum	AAGG	Ara-5	KU8528A	Iraq
T. araraticum	AAGG	Ara-6	KU8593	Iraq
T. araraticum	AAGG	Ara-7	KU8707	Iraq
T. araraticum	AAGG	Ara-8	KU8725	Iraq
T. araraticum	AAGG	Ara-9	KU8831	Iraq
T. araraticum	AAGG	Ara-10	KU8884	Iraq
T. timopheevi	AAGG	Tmp-1	Tmp	Georgia
T. dicoccoides	AABB	Dcd-1	KU1945	Turkey
T. dicoccoides	AABB	Dcd-2	KU1952	Turkey
T. dicoccoides	AABB	Dcd-3	KU1959B	Turkey
T. dicoccoides	AABB	Dcd-4	KU1978B	Turkey
T. dicoccoides	AABB	Dcd-5	KU8935	Turkey
T. dicoccoides	AABB	Dcd-6	KU8736A	Iraq
T. dicoccoides	AABB	Dcd-7	KU8817	Iraq
T. dicoccoides	AABB	Dcd-8	Quatzrin	Israel
T. dicoccoides	AABB	Dcd-9	Yehudiya	Israel
T. dicoccoides	AABB	Dcd-10	Rosh Pinna	Israel
T. durum	AABB	Drm-1	KU127	China
T. durum	AABB	Drm-2	KU3178	Iran
T. durum	AABB	Drm-3	KU3688	Turkey
T. dicoccum	AABB	Dcm-1	KU7309	Ethiopia
T. dicoccum	AABB	Dcm-2	KU10490	Iran
T. dicoccum	AABB	Dcm-3	KU1063A	Spain
T. dicoccum	AABB	Dcm-4	KU3371	Iran
T. aestivum	AABBDD	Ast-1	Chinese Spring	Unknown
T. aestivum	AABBDD	Ast-2	KU1527	Armenia
T. aestivum	AABBDD	Ast-3	KU3097	Iran
T. aestivum	AABBDD	Ast-4	KU3856	Turkey
T. spelta	AABBDD	Spl-1	Spl	Unknown
T. spelta	AABBDD	Spl-2	KU1073	Spain
T. spelta	AABBDD	Spl-3	KU1140	Spain
T. spelta	AABBDD	Spl-4	KU3413	Germany
T. macha	AABBDD	Mch-1	Mch	Georgia
T. macha	AABBDD	Mch-2	KU154	Unknown
T. macha	AABBDD	Mch-3	KU193	Unknown
T. macha	AABBDD	Mch-4	KU1814	Georgia

genome, and the AT content of chloroplast DNA is much higher, most chloroplast microsatellites are $(A/T)_n$ mononucleotide repeats. Using these identified microsatellites, intra- and inter-specific variations were examined in pine tree (Powell et al. 1995b), soybean (Powell et al. 1995a; 1996), rice (Provan et al. 1996; 1997; Ishii and McCouch 2000) and maize (Provan et al. 1999). These studies indicated that the corresponding SSR regions in related species could be amplified by PCR and that chloroplast microsatellites were good markers to clarify phylogenetic relationships.

Wheat is one of the unique crops that went through polyploid evolution. It is well known that hexaploid common wheat (AABBDD) is an allopolyploid species generated from Emmer wheat (AABB) and *Aegilops squarrosa* (DD) (Kihara 1944). RFLP analysis of chloroplast DNA revealed that the cytoplasm of common wheat was derived from that of Emmer wheat (Tsunewaki and Ogihara 1983; Miyashita et al. 1994). However, since a few RFLPs or specific regions were analyzed among these chloroplast genomes, their intraspecific variation as well as their evolution is still not well-explained.

Recently, the complete chloroplast DNA sequence of common wheat (*Triticum aestivum* cv Chinese Spring) was determined by Ogihara et al. (2000). And this provides the opportunities to explore the use of wheat chloroplast microsatellites to analyze relationships among *Triticum* species. In this study, wheat chloroplast microsatellites were identified from the entire chloroplast nucleotide sequence and their allelic diversity was examined using a set of tetraploid and hexaploid *Triticum* species.

Materials and methods

Plant materials

Forty three accessions from *Aegilops* and *Triticum* species which were involved in polyploid evolution were used (Table 1). The phylogenetic relationships among these species, inferred by Kihara (1944) and Lilienfeld (1951), are shown in Fig. 1.

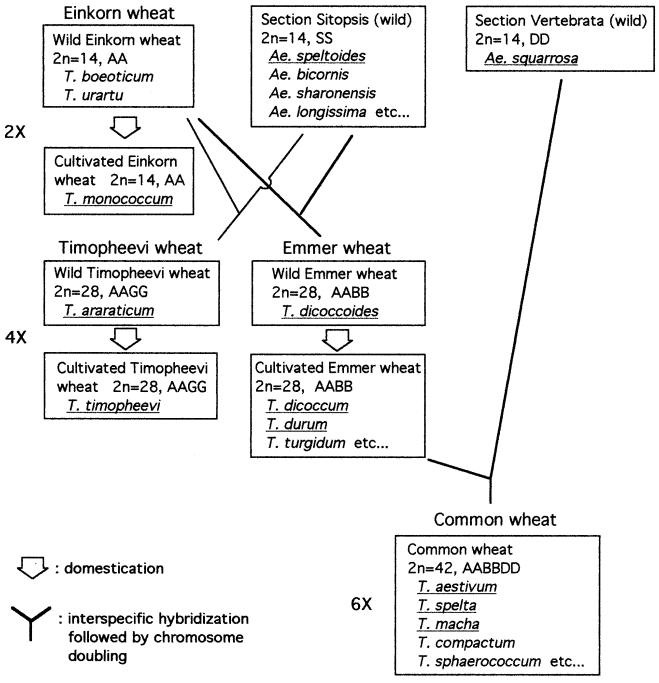

Fig. 1 Phylogenetic relationships among common wheat and its ancestral species. The species used in this study are *underlined*

DNA extraction

Total DNA was extracted from fresh leaves according to the method of Liu et al. (1990). The quality and concentration of extracted DNA were estimated using mini-gel electrophoresis (Sambrook et al. 1989).

Chloroplast microsatellites

Based on the entire chloroplast DNA sequence of common wheat (*T. aestivum* cv Chinese Spring) by Ogihara et al. (2000), 24 chloroplast microsatellites (designated as WCt1–24) having more than ten mononucleotide repeats were identified. No di- or tri-nucleotide repeats were found. For each microsatellite, a pair of primers was designed to produce specific PCR products in the range of 100–200 bp using the PRIMER 0.5 program (E. Lander, Whitehead Institute, Cambridge, Mass., USA). Since WCt20 and 21 are separated by only eight nucleotides, one pair of primers was designed to amplify PCR products containing both microsatellites. The characteristics of the chloroplast microsatellites are listed in Table 2.

PCR amplification and silver staining

PCR was performed in 50-µl reactions containing 100 ng of template DNA, 0.2 µM of each primer, 100 µM of each dNTP, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 1 unit of *Taq* DNA polymerase (TOYOBO, Japan). Amplification was carried out in a PTC100 96U thermocycler (MJ Research, USA) as follows: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 7 min at 72°C for final extension. Amplified products were electrophoresed in 4.0% polyacrylamide gels and the banding patterns were visualized using silver staining as described by Panaud et al. (1996).

Allele scoring

After silver staining of polyacrylamide gels, a cluster of 2–5 discrete bands was apparent for most of the markers. The size (in nucleotides) of the most-intensely amplified band for each microsatellite marker was determined for each genotype based on migration relative to a Chinese Spring standard allele and molecular-weight size markers V and VIII (Boehringer Mannheim, Germany). Chinese Spring was used as a reference for molecular-weight determination because expected allele sizes of PCR products were available for this variety based on the entire chloroplast DNA sequence.

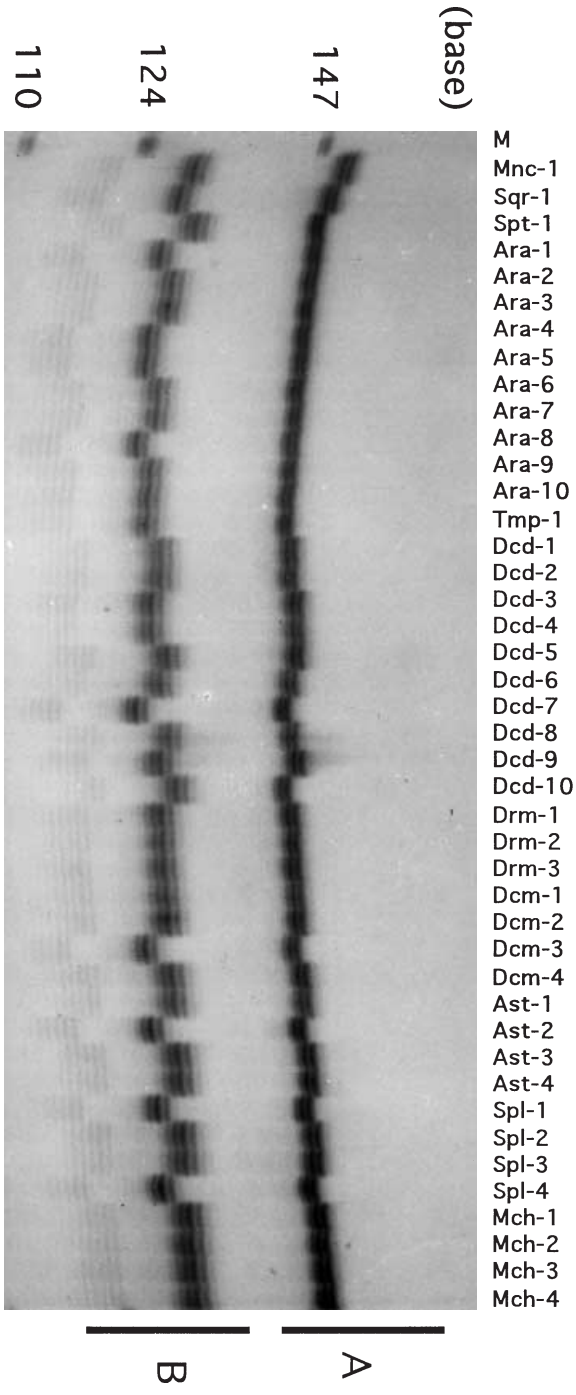

Fig. 2 SSLP band patterns of 43 wheat accessions at two chloroplast microsatellite loci, WCt12 (*A*) and WCt2 (*B*). Both loci were simultaneously detected in a polyacrylamide gel by a single loading of PCR products. All accessions are indicated by codes as shown in Table 1. *M* molecular-weight marker

Table 2 Characteristics of wheat chloroplast microsatellites

Locus	Location (gene)	Repeat	Primer sequence (5′–3′)	Size[a] (bp)	No. alleles	Diversity index (H)
WCt1	Intergenic region (*mat*K–5′*trn*K)	(A) 10	CATCCTTTTCAATCCAAAATCA GATTAGTGCCGGATACGGG	109	2	0.402
WCt2	Intergenic region (*psb*I–*trn*S)	(T) 15	CTTATCTAATGACCCAGGACGG CGAATTGGAAAGAATTCTGACC	132	7	0.716
WCt3	Intergenic region (*psb*C–*trn*S)	(A) 14	TCTGGCTCGGTTATTCCATC TCTAGGAACTATCGAGGGTTCG	148	5	0.602
WCt4	Intergenic region (5′*trn*G–*trn*T)	(T) 11	TCTTCGGAAACGGAAAACC GGATTTCCCATTATGGGTCC	199	5	0.507
WCt5	Intergenic region (ORF29–*trn*C)	(A) 10	TGATATTCTTTCGTGGAGTCCC TGGTATCCAAAGAAAGGTCCC	83	4	0.522
WCt6	Intergenic region (*trn*C–*rpo*B)	(C) 10	TCACAGGCTGCAAAATTCAG GGATAATAATGCTGTTCGGACC	187	4	0.434
WCt7	Coding region (*rpo*C2)	(A) 12	ATCGTTCCCCACAAGACAAG AGGGTTAAATGTTAAATGGGGG	168	1	0.000
WCt8	Intergenic region (*rps*2–*atp*I)	(T) 10	CTTGGGCAACTGCGTAAATT ACCAAGAAAGCACATCAGATCA	150	2	0.045
WCt9	Intergenic region (*atp*I–*atp*H)	(T) 12	CGCAGCCTATATAGGTGAATCC TTGCAACCAAGCAGATTATCC	125	4	0.486
WCt10	Intron (*atp*F)	(A) 12	TGCCCTTTTTTAACCAATGC CATGGTCAGCAAAGTTGTTTC	187	5	0.175
WCt11	Intron (*atp*F)	(A) 14	TTTTATCTAGGCGGAAGAGTCC TCATTTGGCTCTCACGCTC	171	7	0.692
WCt12	Intron (*inf*170)	(T) 10	CGATCCCTATGTAGAAAGCCC AACGAAACCCCTTCTTACCG	149	3	0.595
WCt13	Intergenic region (*trn*F–*ndh*J)	(A) 15	TGAAAATCCTCGTGTCACCA TGTATCACAATCCATCTCGAGG	110	6	0.667
WCt14	Intergenic region (*rpl*23–*psa*I)	(T) 10	TCAACAAGTGACTCGAACTGTG CGTCATGGAATAGGTGTCTCA	199	2	0.089
WCt15	Intergenic region (*psb*E–ORF31)	(T) 14	CAATCTGGTTTTGCCTGGTT ATGGGGTTTTCTATTGATGCC	105	7	0.651
WCt16	Intergenic region (*psb*E–ORF31)	(T) 11	TGGTTGGAGTTCGAAAAAGG AACAAAAGCATTCCACGACC	100	4	0.382
WCt17	Intergenic region (*psb*E–ORF31)	(T) 11	GATCGTTCACTCCAAAAAGAGG ACCCCATTGAATGAAAAAATG	146	4	0.386
WCt18	Intergenic region (*psb*E–ORF31)	(A) 10	TGATTCGGAATTAGGGACTCA GTAAGCATGAAAGAGTTAAATTCCA	199	2	0.045
WCt19	Intergenic region (*rpl*36–*inf*A)	(A) 10	TTTGGAAAAAATAAGTCTCTTCGC GCGTATCGAAGACTCGAAGG	153	4	0.613
WCt20	Coding region (*inf*A)	(T) 10	TTCCATTGGGTAGGGCTTC GTAATCGCCCCGCCTATAGT	178[b]	1	0.000
WCt21	Coding region (*inf*A)	(T) 10	TTCCATTGGGTAGGGCTTC GTAATCGCCCCGCCTATAGT	178 [b]	1	0.000
WCt22	Intergenic region (*rps*8–*rpl*14)	(T) 12	GCAATAGTGTCCTTGCCCAT ACCAAAATAGTTTCATTAGCTCCTG	197	5	0.659
WCt23	Intergenic region (*rpl*14–*rpl*16)	(T) 10	TCCAGAAAGAAAAACCGGG TAGCTGCCAGTAAAATGCCC	108	2	0.500
WCt24	Intergenic region (*ndh*F–*rpl*32)	(A) 14	AAAAATTAAGAATGGGTTTAGTTGG AACAAGAGAGCTGTTTCATCTTTG	189	7	0.586

[a] Expected size in *T. aestivum* cv Chinese Spring [b] PCR products contain both WCt20 and 21 regions

Data analysis

The allelic diversity of chloroplast microsatellites was calculated according to the gene diversity value described by Nei (1987) as follows:

$$H_i = 1 - \sum_{j=1}^{n} x_{ij}^2,$$

where x_{ij} is the frequency of the *j*th allele for marker *i* and summation extends over *n* alleles.

Phylogenetic relationships among *Aegilops* and *Triticum* species were studied based on the similarity of chloroplast microsatellite allele sizes. The ratio of common amplified fragments was used as a similarity index, and calculated according to the following formula:

$F_{ij} = 2B_{ij}/A_{ij}$,

where A_{ij} and B_{ij} are the numbers of total and common fragments observed between the *i*th and *j*th varieties (Nei and Li 1979). Based on the percentages of common fragments, a dendrogram showing similarities among the chloroplast genomes of 43 wheat accessions was constructed by the UPGMA method (Sneath and Sokal 1973).

Results and discussion

Allelic diversity of chloroplast microsatellites among common wheat and its ancestral species

Good amplification was obtained for all 24 chloroplast microsatellites with 43 wheat accessions, as exemplified in Fig. 2. Allele size at each locus was determined based

on the nucleotide length difference from the standard variety, *T. aestivum* cv Chinese Spring. Null alleles were assigned to the varieties giving no amplified products. Among 24 chloroplast microsatellite loci, null alleles were observed in only four marker/accession combinations (0.4%); *T. monococcum* (Mnc-1) and *Ae. squarrosa* (Sqr-1) at WCt14, and *Ae. speltoides* (Spt-1) at WCt 15 and 16. The above results suggest that most of the microsatellite primer pairs have the potential to detect alleles of other wheat species.

Among 43 wheat accessions, polymorphic banding patterns were observed at 21 out of 24 chloroplast microsatellite loci. To characterize the allelic diversity and informativeness of polymorphic microsatellites, the number of alleles and the diversity values were examined for all wheat accessions (Table 2). The number of alleles per polymorphic microsatellite ranged from 2 to 7, with an average of 4.33. Diversity values ranged from 0.05 to 0.72, with an average of 0.47. This value is similar to that of rice chloroplast microsatellites reported by Ishii and McCouch (2000). Among 24 chloroplast microsatellites, three (WCt7, 20 and 21) were located in coding regions and the rest were in non-coding regions, namely, intergenic regions and introns. All three microsatellites in coding regions showed monomorphism among wheat accessions, indicating that the simple sequence length mutation which damages the reading frames might be suppressed in coding regions.

In this study, a chloroplast microsatellite was defined to contain more than ten mononucleotide repeats. The 24 identified chloroplast microsatellites varied in repeat number from 10 to 15 (Table 2). Using the allelic diversity data on 21 polymorphic microsatellite loci (excluding three monomorphic loci located in coding regions) significant correlations ($P<0.01$) were observed between the number of repeats and the number of alleles ($r=0.877$) and between the number of repeats and diversity value ($r=0.588$). These results indicate that the microsatellites with a higher number of repeat motifs have a tendency to show greater allelic diversity, as witnessed by the larger number of alleles per locus and the higher diversity values. Such positive correlations have not been observed in distantly related species, because in the respective species the chloroplast microsatellite motifs were usually interrupted and different kinds of mutations have accumulated in the regions flanking the microsatellites (Bryan et al. 1999; Weising and Gardner 1999; Ishii and McCouch 2000).

Chloroplast haplotypes detected among common wheat and its ancestral species

Among 43 wheat accessions, polymorphic banding patterns were observed at 21 out of 24 chloroplast microsatellite loci. Since the chloroplast genome does not genetically recombine, allele size variation at individual chloroplast microsatellite loci can be combined to obtain chloroplast haplotypes. Based on the size variation at all chloroplast microsatellite loci, a total of 24 chloroplast haplotypes were identified among common wheat and its ancestral species (Table 3). The overall haplotypic diversity value was 0.912. In this study, more than three accessions were examined for seven species (*T. araraticum*, *T. dicoccoides*, *T. durum*, *T. dicoccum*, *T. aestivum*, *T. spelta* and *T. macha*). All three accessions of *T. durum* shared the same chloroplast haplotype, whereas each of the other species possessed at least two chloroplast haplotypes.

Intraspecific variation of chloroplast genomes in *T. dicoccoides* and *T. araraticum* detected by SSR and RFLP analyses

Ten accessions each of the wild tetraploid wheats, *T. dicoccoides* and *T. araraticum*, were used to examine intraspecific variation of the chloroplast genomes. Based on the 24 chloroplast microsatellite patterns, the chloroplast genomes were classified into nine and five chloroplast haplotypes for *T. dicoccoides* and *T. araraticum*, respectively. According to Mori (1991), three chloroplast genome types were observed among 27 *T. dicoccoides* accessions by chloroplast RFLP analysis using four restriction enzymes, and no intraspecific variation was found among 27 accessions of *T. araraticum*. Miyashita et al. (1994) studied restriction map variation of 13 sites in two 5–6-kb chloroplast DNA regions, and classified 14 *T. dicoccoides* and ten *T. araraticum* accessions into three and two types, respectively. Compared to these chloroplast RFLP analyses, the microsatellites are very powerful to identify or classify the chloroplast genome types. This result suggests that the simple sequence repeat length mutation rate in chloroplast microsatellites is higher than the rate of base substitution and insertion/deletion events.

Comparison of genetic diversity between chloroplast microsatellite and nuclear RFLP markers

The chloroplast genome is known to have a conservative nature (Wolfe et al. 1987); however, the chloroplast microsatellites showed high variability. In order to examine the level of variability, the genetic diversity explained by chloroplast microsatellites and nuclear RFLP markers was compared. Previously, Mori (1991) and Mori et al. (1995; 1997) carried out nuclear RFLP analysis on tetraploid wheat species which included 22 plant materials used here, i.e. ten *T. araraticum* (Ara-1 – Ara-10) and ten *T. dicoccoides* (Dcd-1 – Dcd-10) accessions, one *T. timopheevi* (Tmp-1) and one *T. durum* (Drm-1) accession. Therefore, the genetic diversity was calculated based on the results for the types of fragment patterns digested with a single enzyme (*Hin*dIII) using the following 23 polymorphic probes: TAG13, 221, 317, 341-2, 398, 510, 538, 539, 549, 577, 587, 609, 653, 694, 708, 744, 762, TAC64-1, 64-2, 72, 76, 77, 111 (see Liu and

Table 3 Size variation of amplified fragments at 24 chloroplast microsatellite loci found among 24 wheat chloroplast haplotypes

Haplo-type	Locus and size in bp[a]																								Accession[b]
	WCt 1	WCt 2	WCt 3	WCt 4	WCt 5	WCt 6	WCt 7	WCt 8	WCt 9	WCt 10	WCt 11	WCt 12	WCt 13	WCt 14	WCt 15	WCt 16	WCt 17	WCt 18	WCt 19	WCt 20	WCt 21	WCt 22	WCt 23	WCt 24	
	109	132	148	199	83	187	168	150	125	187	171	149	110	199	105	100	146	199	153	178	178	197	108	189	
1	0	–2	6	–2	1	–3	0	0	–1	7	–5	1	–4	N	–4	1	0	0	0	0	0	2	–1	–8	Mnc-1
2	0	–3	–3	–1	0	–3	0	–1	–3	9	–5	0	–4	N	–5	0	–2	–1	–2	0	0	2	–1	15	Sqr-1
3	1	0	–2	0	3	0	0	0	–3	0	1	–1	4	0	N	N	0	0	0	0	0	–9	–1	–4	Spt-1
4	1	–4	–2	–1	0	–1	0	0	–2	0	5	–1	2	0	3	1	–1	0	–1	0	0	2	–1	–2	Arr-1,4,8
5	1	–4	–2	–1	0	–1	0	0	–2	0	4	–1	2	0	3	1	–1	0	–1	0	0	2	–1	–2	Arr-5
6	1	–2	–1	0	0	–1	0	0	–3	0	4	–1	2	0	3	1	–1	0	–1	0	0	–1	–1	–2	Arr-2
7	1	–2	–2	7	0	–1	0	0	–2	0	4	–1	2	0	3	1	0	0	–1	0	0	–1	–1	–2	Tmp-1
8	1	–2	–2	–1	0	0	0	0	–2	0	4	–1	–2	0	3	0	0	0	–1	0	0	0	–1	–1	Arr-3,6,9,10
9	1	–2	–2	–1	0	0	0	0	–2	0	4	–1	2	0	3	0	0	0	–1	0	0	0	–1	–1	Arr-7
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Dcd-1, Drm-1,2,3, Dcm-4, Ast-1,3,4, Spl-2,3
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Dcd-2, Dcm-2, Mch-4
12	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mch-1,2
13	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mch-3
14	0	0	0	0	1	0	0	0	0	0	–1	0	0	0	0	0	1	0	0	0	0	0	0	0	Dcd-6
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–1	–1	0	0	0	0	0	–1	0	Dcm-1
16	0	1	0	0	2	0	0	0	0	0	–1	0	0	0	0	0	0	0	0	0	0	0	0	1	Dcd-8
17	0	0	0	0	2	0	0	0	0	0	–1	1	0	0	0	0	0	0	0	0	0	1	0	0	Dcd-9
18	0	–1	0	0	1	–1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	–1	0	0	Dcd-3,4
19	0	1	0	0	1	1	0	0	0	1	0	1	–1	0	–1	0	0	0	0	0	0	2	0	0	Dcd-5
20	0	–3	0	1	2	0	0	0	0	0	0	–1	–4	0	2	–1	1	0	1	0	0	–1	–1	0	Dcd-7
21	0	2	–1	–1	1	–1	0	0	0	0	2	–1	–4	0	2	0	1	0	1	0	0	–1	–1	–1	Dcd-10
22	0	–3	–1	0	1	0	0	0	0	0	–1	–1	–4	0	2	0	0	0	1	0	0	–1	–1	–1	Dcm-3
23	0	–3	–1	0	1	0	0	0	0	0	–1	–1	–4	0	2	0	0	0	1	0	0	–1	–1	0	Spl-1,4
24	0	–3	–1	0	1	0	0	0	0	–1	–1	–1	–4	0	2	0	0	0	1	0	0	0	–1	0	Ast-2

[a] Allele size was designated as the nucleotide length difference (+/– for longer/shorter) from *T. aestivum* cv Chinese Spring allele size. N: null allege (no amplification)
[b] All accessions are indicated by codes as shown in Table 1

Table 4 Range and average values of the number of alleles and the diversity index for chloroplast microsatellite and nuclear RFLP markers observed among tetraploid wheat accessions

Accession	Marker	No. markers	No. alleles[a]			Diversity index (H)		
			Min.	Max.	Ave.	Min.	Max.	Ave.
All tetraploid accessions (22 accessions)	CtMS[b]	18	2	7	3.56	0.09	0.79	0.57
	RFLP[c]	23	2	15	6.44	0.25	0.91	0.67
T. dicoccoides (10 accessions)	CtMS[b]	18	1	5	2.61	0.00	0.74	0.40
	RFLP[c]	23	1	8	3.96	0.00	0.86	0.45
T. araraticum (10 accessions)	CtMS[b]	18	1	3	1.67	0.00	0.58	0.25
	RFLP[c]	23	1	6	2.22	0.00	0.80	0.26

[a] Corresponding to the number of banding patterns for RFLP markers
[b] Polymorphic chloroplast microsatellites among 22 tetraploid accessions
[c] Polymorphic nuclear RFLP markers among 22 tetraploid accessions

Tsunewaki 1991 and Mori et al. 1995 for probe information). Table 4 gives the range and average values for the number of fragment patterns and the diversity index among 22 tetraploid, ten *T. araraticum* and ten *T. dicoccoides* accessions. As for chloroplast microsatellites, polymorphisms were detected among tetraploid accessions at 18 out of 24 loci, and the allelic diversity was also calculated as shown in Table 4. Although the number of alleles for nuclear RFLP markers was higher than that of chloroplast microsatellites, similar diversity values were observed for both markers. This indicates that the chloroplast microsatellites had fewer alleles but gave almost the same level of informativeness as the nuclear RFLP markers.

Phylogenetic relationships among cultivated and wild wheat species as suggested by the chloroplast SSR analysis

Microsatellites are useful markers to detect high levels of allelic variation among species or accessions. However, Doyle et al. (1998) reported that phylogenetic evaluation using a few chloroplast microsatellites does not always reflect species relationships, because of the size homoplasy which occurs in amplified bands with the same molecular weight containing different internal mutations. In this study, a total of 24 chloroplast microsatellites were surveyed among 43 wheat accessions, and polymorphisms were observed at 21 chloroplast microsatellite loci with high average diversity value of 0.47. Since these polymorphic loci were from different regions distributed in the entire chloroplast genome, the similarity between accessions or haplotypes may not be seriously affected by size homoplasy at one or two loci (see Table 3).

In order to examine the phylogenetic relationships among wheat species, a similarity matrix between 43 accessions was calculated based on the pairwise-scoring of alleles, and a dendrogram showing similarities among chloroplast genomes of 43 wheat accessions was constructed (Fig. 3). Among three diploid species, *Ae. speltoides* (Spt-1) shows a closer relationship to tetraploid (AABB and AAGG) and hexaploid (AABBDD) wheat species. This is in agreement with the fact that *Ae. speltoides* was the possible cytoplasm donor of these wheat species (Ogihara and Tsunewaki 1988; Tsunewaki 1996). Timopheevi wheat species were clearly distin-

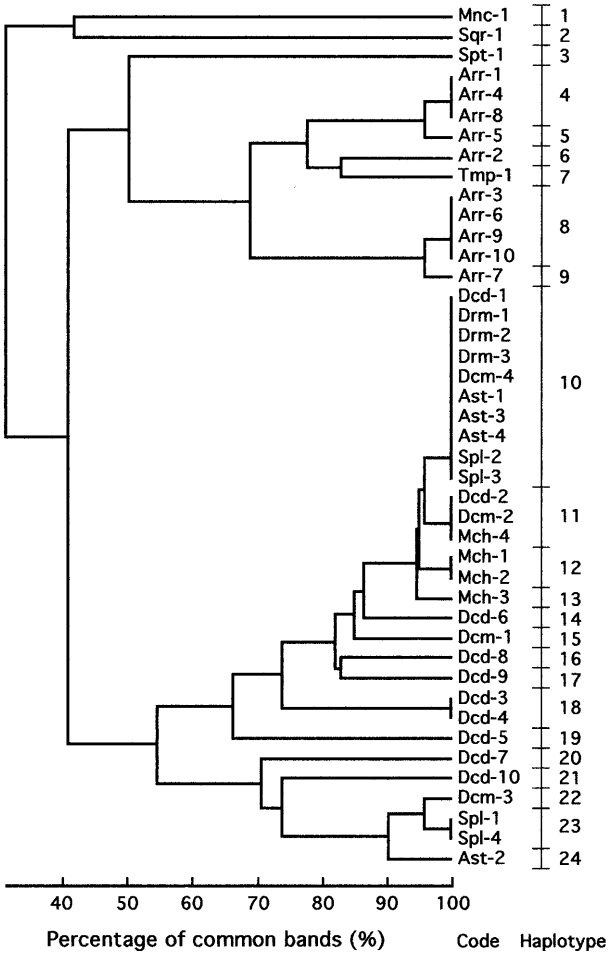

Fig. 3 A dendrogram showing similarities among chloroplast genomes of 43 wheat accessions based on chloroplast SSLP analysis. All accessions are indicated by codes as shown in Table 1

guished from Emmer and common wheat species. Wild Timopheevi wheat, namely, *T. araraticum*, was divided into two groups, and cultivated *T. timopheevi* clustered with one of them. Since only a single accession of *T. timopheevi* was used in this study, the phylogenetic relationships among Timopheevi wheat is still unclear. More accessions of *T. timopheevi* should be analyzed to clarify their domestication pathway.

As for Emmer and common wheat species, although *T. dicoccoides* showed wide intraspecific variation, two main groups were observed: one consisted of *T. dicoccoides* (Dcd-7 and 10), *T. dicoccum* (Dcm-3), and common wheat (Ast-2, Spl-1 and 4) accessions having haplotypes 20–24, and the other contained the rest of the accessions possessing haplotypes 10–19. It is of interest that the two main groups include a series of wild and cultivated species from tetraploid to hexaploid, and common wheat has two discrete chloroplast genome types (comprising haplotypes 10–13 and haplotypes 23–24, respectively). These findings have never been pointed out by earlier studies using other molecular markers, such as RAPD, nuclear and organellar RFLP, and nuclear SSLP markers (Joshi and Nguyen 1993; Plaschke et al. 1995; Mori et al. 1997; Wang et al. 2000). The results of the present study indicate that the two types of chloroplast genomes of common wheat might have independently originated from the corresponding types of wild and cultivated Emmer wheat species.

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