

Evidence for the origin of the B genome of bread wheat based on chloroplast DNA

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Abstract: The hexaploid bread wheat *Triticum aestivum* L. (AABBDD) is thought to have originated through one or more rare hybridization events between *Aegilops tauschii* (DD) and the tetraploid Emmer wheat *T. turgidum* subsp. *dicoccon* (AABB). The progenitor of the A genome of *T. aestivum* has generally been accepted to be *T. urartu*. The origin of the B genome, however, is controversial and still relatively unknown. Research has found the B genome in *T. turgidum* to be closely similar to the S genome in section *Sitopsis* of *Aegilops* L.; the *Sitopsis* diploid species were thus proposed as the probable maternal parent in the original cross that resulted in the tetraploid *T. turgidum*. Moreover, the donor of the cytoplasm of *T. turgidum* and *T. aestivum* was also the donor of most, if not all, of the B genome chromosomes. The present study attempts to investigate the polymorphism of chloroplast DNA between *T. aestivum* and 8 different *Aegilops* species using cleaved amplified polymorphic sequence (CAPS) and sequencing on 28 chloroplast loci in order to identify the chloroplast donor (B genome donor) of bread wheat. A phylogenetic tree based on the data generated demonstrates that *Ae. speltoides* was distinct from the other *Aegilops* species analyzed and the most closely related to bread wheat. It can be concluded that *Ae. speltoides* may be the chloroplast donor to bread wheat and the donor of its B genome. Understanding hexaploid wheat origin would further its genetic improvement and is also important for the artificial development of synthetic forms.

Key words: *Aegilops*, CAPS, chloroplast DNA, identification, sequencing

Introduction

Wheat (*Triticum* L.) is an annual plant that belongs to the grass family Poaceae, tribe Triticeae, and subtribe Triticineae. It is thought to have originated on the Eurasian continent, a starting point from which man spread it throughout the world, including China and central Europe. Wheat is one of the earliest domesticated crop plants in the Pre-Pottery Neolithic Near East (Lev-Yadun et al. 2000). The center of its domestication is widely accepted to be somewhere in the Middle East (Anikster and Wahl 1979). It is the

world's most widely cultivated food crop, followed by rice and maize (Gulbitti-Onarici et al. 2009), and one of the oldest and most important of the cereal crops (Harlan 1992), producing the highest global grain production of any crop (Lamoureux et al. 2005).

The genus *Triticum* exists as a polyploid series of diploid, tetraploid, and hexaploid species complexes (Provan et al. 2004). Of special cultural and economic importance are the tetraploid durum wheat *T. turgidum* L. and the hexaploid bread wheat (common wheat) *T. aestivum* L. (Baum et al. 2009). *T.*

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aestivum L. is by far the most important staple crop in the world. The economic significance of this crop has attracted attention for a long time and stimulated intense interest in the determination of its ancestral diploid genome donors (Gulbitti-Onarici et al. 2009).

The origin of bread wheat and its genome donors

Salamini et al. (2002) state that understanding the origin of hexaploid wheat would further its genetic improvement. Moreover, knowledge about the role diploid species play in the evolution of polyploid wheats is important for the development of artificial synthetic forms (Gulbitti-Onarici et al. 2009).

T. aestivum (AABBDD) is derived from the 3 homologous genomes, A, B, and D, each of which contributes 7 pairs of chromosomes to the wheat's total genome ($2n = 6x = 42$). This species is widely believed to have originated between 7000 and 10,000 years ago through 1 or more rare hybridization events (Zohary and Hopf 2000) between a diploid species ($2n = 2x = 14$, DD) of the genus *Aegilops* L. (a sister genus to *Triticum* that belongs to the same hierarchical classification) and a wild tetraploid species (AABB). Such an event would have initially generated a sterile triploid (ABD) and subsequent chromosome doubling could have led to the production of the allohexaploid. It is most likely that the tetraploid parent was the tetraploid wild Emmer *T. turgidum* subsp. *dicoccon* (AABB) (Kihara 1944), since the geographical distribution of subsp. *dicoccoides* does not overlap with that of *Ae. tauschii* (Zohary and Hopf 1988).

The D genome progenitor of *T. aestivum* is generally accepted to be *Ae. tauschii* Coss. (syn. *Ae. squarrosa* auct. non L.) (Petersen et al. 2006; Zhang et al. 2008). Regarding the A genome of *T. aestivum*, there has been little debate regarding its origin, and 3 species were suggested as the A genome donor to polyploid wheats: *T. monococcum* L. (e.g. Sourdille et al. 2001), *T. urartu* Thum. ex Gandilyan (e.g. Gulbitti-Onarici et al. 2007), and *T. boeoticum* (*T. monococcum* var. *boeoticum*) (e.g. Gulbitti-Onarici et al. 2009).

In polyploid wheat, the donor of the B genome has been the most controversial and is still relatively unknown, in spite of a large number of attempts to identify the parental species (Huang et al. 2002). This

may be associated with the higher diversification rate of the B genome compared to the A(u) genome in the polyploid wheats (Petersen et al. 2006), the incomplete chromosome pairing between B genome chromosomes and any diploid species (Blake et al. 1999), and the fact that the B genome is relatively diverged from its putative diploid progenitors (Talbert et al. 1995).

Two *Triticum* species were proposed as the B genome donors of bread wheat. The first of these is *T. villosum* (L.) ($2n = 14$, VV), a suggestion based on isozymes, seed storage proteins, and a careful comparison of the restriction fragment patterns of both the chloroplast and mitochondrial DNAs (hereafter cpDNA and mtDNA, respectively) (Montebove et al. 1987; Shu et al. 1993). *T. urartu* has also been proposed as a potential donor based on protein electrophoresis and cytological and geographical distribution studies (Johnson 1972; Johnson and Dhaliwal 1976).

Several studies have shown that the B genome in *T. turgidum* and *T. aestivum* is closely similar to the S genome in section *Sitopsis* (Huang et al. 2010). Therefore, one or more of the *Sitopsis* species were frequently proposed as the B genome donor to polyploidy wheats, including *Ae. bicornis* (e.g. von Buren 2001); *Ae. longissima* (e.g. Ogihara and Tsunewaki 1982); *Ae. searsii* and *Ae. sharonensis*, considered by Kihara (1954) and Tanaka (1955) to be 2 of the *Ae. longissima* variants (e.g. Feldman 1978; Baum et al. 2009); and *Ae. speltooides* (e.g. Salina et al. 2006), for which the most positive evidence has been accumulated.

Based on cytoplasmic studies (e.g. Vedel et al. 1978), the *Sitopsis* diploid species were proposed as the probable maternal parent in the original cross that resulted in the tetraploid *T. turgidum*. Several other investigations by Sarkar and Stebbins (1956), Sasanuma et al. (1996), Maestra and Naranjo (1998), and Provan et al. (2004) also confirmed that the cytoplasm of *T. aestivum* is similar to the cytoplasm of the S-type of the 5 *Sitopsis* species of *Aegilops*. Thus, the donor of the cytoplasm of *T. turgidum* and *T. aestivum* was also the donor of most, if not all, of the B genome chromosomes (Bowman et al. 1983) thought to have been modified at the tetraploid level (Johnson and Dhaliwal 1978) and showing a very

high divergence compared to the A and D genomes (Sourdille et al. 2001).

Sequencing and restriction enzyme digestion of cpDNA have been regarded as useful tools for studying interspecific phylogenetic relationships in plants (Kucuk et al. 2006; Yao et al. 2008). For this reason, the present study investigated the polymorphism of cpDNA between *T. aestivum* and 8 *Aegilops* species using cleaved amplified polymorphic sequence (CAPS) and direct sequencing on 28 chloroplast coding and noncoding loci using universal primers. This was done in an attempt to identify the chloroplast donor (B genome donor) of bread wheat.

Materials and methods

Plant material and DNA extraction

Dry seeds of land race accessions of *T. aestivum* and 8 *Aegilops* species (Table 1) were provided by the Genetic Resources Unit (GRU) of the International Center for Agricultural Research in the Dry Areas (ICARDA) in Aleppo, Syria. Information about the accessions used and their geographic origins is given in Table 1. A sample of the diploid species *Amblyopyrum muticum* (once regarded as a species of *Aegilops*, *Ae.*

mutica Boiss.) was used as an outgroup; it is now fairly widely regarded as the only representative of the monospecies genus *Amblyopyrum* (van Slageren 1994).

For DNA extraction, fresh leaves were collected from plants 2-3 weeks after seed germination. DNA was isolated using the DNeasy™ Plant Mini Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions. Recovered DNA pellets were dried under the laminar flow and then resuspended in 150 µL of double-distilled and sterilized water. DNA was quantified using a GeneQuant spectrometer (Amersham Biosciences) and the concentration of all samples was set to 10 ng µL⁻¹.

Polymerase chain reaction (PCR)

PCR was carried out to amplify a total of 28 chloroplast coding and noncoding loci using universal primer pairs. Of these, there were 22 loci that were targeted with primers developed by Haider (2003). Primers that target the chloroplast noncoding regions *trnT-L*, *trnL* intron, and *trnL-F* (Taberlet et al. 1991) were also used. The remaining 3 loci were introns of *trnK* and *16S* and the *psbC-trnS* intergenic spacer.

Table 1. Accessions of *Aegilops* species, *A. muticum*, and *T. aestivum* used in this study; taxonomic treatment according to van Slageren (1994).

Section	Genus and species	ICARDA accession used
<i>Vertebrata</i> Zhuk. emend. Kihara	<i>Ae. tauschii</i> Coss. (syn. <i>Ae. squarrosa</i> auct. non L.)	IG. 46802, Turkey
<i>Aegilops</i> L.	<i>Ae. umbellulata</i> Zhuk.	IG. 47511, Syria
<i>Comopyrum</i> (Jaub. & Spach) Zhuk.	<i>Ae. comosa</i> Sm. in Sibth. et Sm.	IG. 107267, Greece
	<i>Ae. uniaristata</i> Vis.	IG. 47097, Turkey
<i>Cylindropyrum</i> (Jaub. & Spach) Zhuk.	<i>Ae. markgrafii</i> (Greuter) Hammer (syn. <i>Ae. caudata</i> auct. non L.)	IG. 49073, Lebanon
	<i>Ae. speltooides</i> Tausch	IG. 48433, Syria
	<i>Ae. longissima</i> Schweinf. & Muschl.	IG. 48613, Palestine
<i>Sitopsis</i> (Jaub. & Spach) Zhuk.	<i>Ae. bicornis</i> (Forssk.) Jaub. & Spach	IG. 46854, Turkey
	<i>Amblyopyrum muticum</i> (Boiss.) Eig	IG. 47907, Turkey
	<i>T. aestivum</i> L. (ssp. <i>aestivum</i> L. emend. Thell.)	IG. 43266, Pakistan

PCR was performed in 0.2-mL microtubes (Greiner Bio-One, USA) using an Eppendorf thermocycler. Each reaction contained 10× PCR buffer (Eurobio), 10× MgCl₂ (50 mM, Eurobio), forward primer (15 μM, Invitrogen), reverse primer (15 μM, Invitrogen), dNTPs (10 mM, Mix Roche), and Taq polymerase (5 U μL⁻¹, Eurobio). DNA was added to each PCR reaction at a rate of 20 ng and the total volume was adjusted with double-distilled H₂O to 20 μL. For 35 cycles, PCR reactions were subjected to 94 °C for 30 s for DNA denaturation, 46.6-58.5 °C for 1 min for the annealing of primers, 72 °C for 1 min for the extension of the target chloroplast region, and 72 °C for 5 min for the final extension. For the visualization of PCR products, 2 μL of each PCR product was loaded into 1.5% agarose gel that was run at 120 V for 30 min.

Cleaved amplified polymorphic sequence (CAPS)

For CAPS analysis of all *Aegilops* species, *A. muticum*, and *T. aestivum*, 11 chloroplast loci were amplified as described above. These were: *psbE&psbF*, *rpoA.1*, *ndhF.1*, *ndhF.2*, *psbH*, *rpl2&trnH.2*, *psbK*, *psbI&trnS.1*, *rpl23&rpl2.1*, *rpl23&rpl2.2* (Haider

2003), and the *trnL* intron and the intergenic spacer *trnT-L* of Taberlet et al. (1991).

Restriction digests were performed on PCR amplicons generated from the targeted loci for single nucleotide polymorphism (SNP) detection in those loci. Each marker exploited a potentially variable SNP positioned within the amplicon and in which one allelic state coincided with a recognition site of a restriction enzyme. PCR products (1-5 μL) were digested according to the instructions of the manufacturer (Fermentas) using 41 single-cut endonucleases (Table 2). Digested fragments were separated by electrophoresis on 2% agarose gel that was run at 100 V for 2 h in 1× TAE buffer and visualized under UV lights using a gel documentation system (GDS8000, UVP).

Sequencing

For sequencing, which was performed in the laboratories of the Department of Botany at the University of Reading in Reading, UK, 17 chloroplast loci were amplified by PCR in all species samples. Out of these, 13 were targeted using primers developed by Haider (2003). These were: 1) *psbC*, 2) *23,4.5S&5S*, 3) *psbE-F&orf38&40*, 4) *rpoA.2*, 5) *rbcL.1*, 6) *clpP.1*, 7)

Table 2. The chloroplast loci and enzymes used for the restriction of amplification products.

Chloroplast locus	Restriction enzymes					
<i>psbE&psbF</i>	HinfI	HpaII	AvaII			
<i>rpoA.1</i>	EcoRV	AvaII				
<i>ndhF.1</i>	EcoRII	HinfI				
<i>ndhF.2</i>	HindIII	BamHI	NdeII	HpaII	DdeI	
<i>psbH</i>	XbaI	HpaII	EcoRI	EcoRII	HaeIII	
<i>rpl2&trnH.2</i>	HhaI	EcoRII	XbaI	RsaI		
<i>psbK,psbI,&trnS.1</i>	ScaI	HindIII	KpnI			
<i>rpl23&rpl2.1</i>	HhaI	HaeII	NsiI	ScaI	NdeII	
<i>rpl23&rpl2.2</i>	AvaII	EcoRII	NdeII			
<i>trnT-L</i>	TruII	DdeI	TaqI	AluI		
<i>trnL</i> intron	DdeI	MvaI	TaqI	TruII	AluI	

clpP.2, 8) *rps4*, 9) *orf62*, 10) *rpL16(3' end)* & *rpL14(5' end)*, 11) *petB* & *petD*, 12) *psbE-psbF-psbL-psbJ*, and 13) *rp123* & *rp12.2* (*rpL2* intron). Introns of *trnK* (FP: 5' GGAAAAAAGAGCATGTCG 3' and RP: 5' CAACCCAATCGCTCTTTTG 3') and *16S* (FP: 5' ATTGCGTCGTTGTGCCTGG 3' and RP: 5' GATACGTTGTTAGGTGCTCC 3') and the intergenic spacers *psbC-trnS* (FP: 5' CAAGGACCTACTGGTTTAGG 3' and RP: 5' AGGAGTCATGGAAAGAACAGG 3') and *trnL-F* (Taberlet et al. 1991) were also sequenced.

Amplification products generated were purified using either a NucleoSpin or QIAGEN kit prior to subjection to cycle sequencing. Sequencing was performed with the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit produced by PE Applied Biosystems. Each reaction contained dye terminator version 1.0, purified PCR product (3.5 µL), forward or reverse primer (1 µM), and 0.9 µL of sterile nanopure water, to bring the total volume to 10 µL. A PCR-100 Peltier Thermal Cycler was used to run a sequencing program of 30 or 35 cycles, each of which started with 96 °C for 30 s, followed by 50 °C for 15 s and ending with 60 °C for 4 min. Cycle sequencing products were purified using Sephadex (G-50) columns. Sequences generated for each of the chloroplast regions were aligned to resolve nucleotide variation among targeted species. To verify that each primer set amplified the intended locus of the chloroplast genome, each sequence obtained for every chloroplast locus amplified was subjected to BLASTN alignments on the NCBI database (<http://blast.ncbi.nlm.nih.gov>).

Phylogeny construction

The combined data generated from CAPS and the sequencing for all chloroplast loci targeted in *A. muticum*, *T. aestivum*, and the 8 diploid species of *Aegilops* were used to construct a phylogeny of those species. The SeqMan, EditSeq, and MegAlign programs of the software packages DNASTAR Lasergene and MacClade were used to assemble, edit, and align the sequences for SNP (base substitution, insertions, and deletions) detection. SNP markers detected in generated sequences were scored directly into a spreadsheet as present (1) and absent (0) for SNPs. Similarly, CAPS data were scored as (1) when the amplification product of a targeted locus

was positively restricted and as (0) when negatively restricted. When there were no SNPs in the sequence pile-up of a certain locus or a positive restriction with no polymorphism among species targeted in the restriction profile using the same enzyme, the locus (for sequencing) or locus-enzyme combination (for CAPS) was scored as (1) for all species in the matrix. A genetic similarity matrix was computed according to the Jaccard similarity index (Jaccard 1908). In order to reveal genetic relationships among the species analyzed, a dendrogram was constructed by employing the unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal 1973) using PowerMarker software (Liu and Muse 2005) and 100 bootstrap iterations applied to evaluate the reproducibility of the ordination in a statistical way. The species *A. muticum* was considered to be an outgroup for the dendrogram construction.

Results

Amplification of chloroplast loci in *T. aestivum* and *Aegilops* species

All universal primer pairs used to target the 28 chloroplast loci generated PCR amplicons of the appropriate size when applied to template DNA from all of the species analyzed. Generally, a single, clear band was observed when the PCR products generated were fractionated through agarose gels and visualized by ethidium bromide staining. In addition, the amplicons produced were of the same size as those reported by Haider (2003) and Taberlet et al. (1991). Furthermore, there was no size variation among species analyzed for 27 out of 28 loci amplified (Figure 1). The exception to this rule was the amplicons of *trnL-F* (Figure 2), where *Ae. comosa* and *Ae. markgrafii* yielded amplicons of around 460 bp, whereas those generated from all remaining *Aegilops* species, *T. aestivum*, and *A. muticum* were approximately 410 bp.

CAPS analysis

The restriction of amplicons of each locus targeted using 2-5 endonucleases was either complete or absent. There was failure to restrict across all 10 species for 7 (*ndhF.1*-EcoRII, *psbH*-EcoRI and -EcoRII, *rp12* & *trnH.2*-HhaI and -EcoRII, *rp123* & *rp12.1*-HhaI, and *rp123* & *rp12.2*-EcoRII) of the

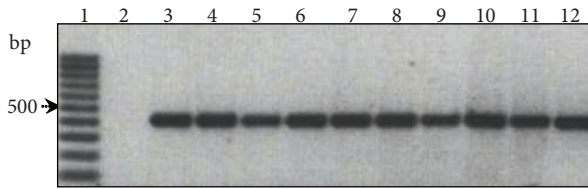


Figure 1. The amplification product generated for the 23S,4.5S&5S locus. Lane 1, 100 bp DNA ladder; lane 2, a negative control; lanes 3-12, products of all species analyzed.

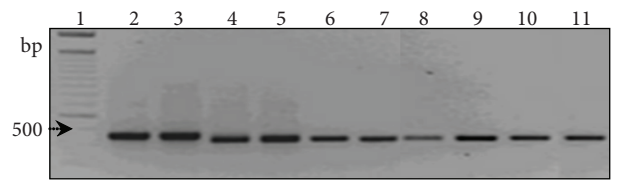


Figure 2. The amplification products generated using *trnL-F* primers. Lane 1, 100 bp DNA ladder; lanes 2 and 3, products of *Ae. comosa* and *Ae. markgrafii*; lanes 4-11, products of remaining species.

41 locus-enzyme combinations tested (Table 2), while 32 of the remaining combinations had a positive and identical restriction profile in all samples. In the 2 remaining combinations, *psbE*&*psbF*-HpaII resulted in identical and positive restrictions in *Ae. speltoides* and *T. aestivum* but failed to restrict in the remaining species. In the other combination, however, *trnT-L*-TaqI, the enzyme restricted only in *Ae. tauschii*.

Sequencing of chloroplast loci

The purification of recovered PCR products increased their concentration and resulted in the removal of any traces of the primer dimers. Electropherogram traces generated after cycle sequencing were clean in both the forward and the reverse directions for all of the loci targeted. BLASTN, carried out in 2010 on representative sequences obtained for every locus sequenced, resulted in the predicted homologous sequences being identified from *T. aestivum*, *Aegilops*, or, when not available, *Hordeum vulgare*. Running a BLASTN search on the sequence of *rpoA.2* generated from *Ae. speltoides* used in the present study, for example, resulted in a homologous sequence that has been identified as “*Aegilops speltoides* RNA polymerase alpha subunit (*rpoA*) gene, complete cds; chloroplast gene for chloroplast product” (Accession: AY115909.1) with a 100% level of certainty of match.

The high level of sequence conservation of targeted loci among species meant that sequence alignment across the 10 species was a relatively simple task using the ClustalX software. Conserved regions along these alignments were displayed through the GenDoc program, which facilitated the detection of SNPs in these loci.

Generally, very little polymorphism was observed among species for the loci sequenced. No

SNP variation was observed in 3 of the targeted loci, namely *psbE-F*&*orf38*&*40*, *clpP.1*, and *psbE-psbF-psbL-psbJ*. The highest level of variation was displayed by *trnL-F*, the *trnK* intron, and *petB*&*D*. These 3 loci included 2 types of SNP variation: deletion/insertion and nucleotide substitution. SNPs detected among species in sequence alignments of those loci are summarized in Table 3. Size variation observed among species for amplicons of *trnL-F* was confirmed by sequencing, since *Ae. markgrafii* and *Ae. comosa* yielded amplicons that were 53 bases longer than those of the other species.

Phylogeny of *Aegilops* species and *T. aestivum*

Sequences generated for the 17 chloroplast loci analyzed here were combined to make a data set of about 6650 bp and used as a basis, together with CAPS data, for the construction of a similarity matrix and phylogeny of bread wheat and the 8 diploid *Aegilops* species.

The similarity matrix (Table 4) had similarity values that ranged from 0.54 (between samples of *T. aestivum* and *Ae. markgrafii*) to 0.96 (between samples of *Ae. bicornis* and *Ae. longissima*). The mean of the similarity values for all of the samples analyzed ranged from 0.61 (for *Ae. speltoides*) to 0.77 (for *Ae. bicornis*). The second highest similarity value mean was that of the *Ae. longissima* sample (0.26).

In the phylogenetic tree constructed (Figure 3), 2 main clusters (bootstrap 100%) were observed. The first cluster included the outgroup *A. muticum* and all *Aegilops* species (bootstrap 99%) other than *Ae. speltoides*. The closest species to the outgroup was *Ae. markgrafii* (bootstrap 43%). In this cluster, *Ae. bicornis* and *Ae. longissima* formed a single clade as sister species (bootstrap 98%), differing from each other only in 2 characters (nucleotide substitutions).

Table 3. A summary of SNPs detected among species in sequence alignments of loci *trnL-F*, *trnK* intron, and *petB&D*.

Chloroplast locus	SNPs (base substitutions/indels)	Position in the edited sequence alignment/bp	Type of polymorphism
<i>trnL-F</i>	A deletion of 4 bases (ATTT)	217	Specific to <i>Ae. bicornis</i> and <i>Ae. longissima</i>
	A deletion of 5 bases (CAATG)	23	Specific to <i>Ae. speltoides</i> and <i>T. aestivum</i>
	A base substitution (A to T)	229	Separates <i>Ae. bicornis</i> , <i>Ae. uniaristata</i> , <i>Ae. markgrafii</i> , <i>Ae. muticum</i> , and <i>Ae. speltoides</i> from the remaining species
	A base substitution (G to A)	141	Separates <i>Ae. tauschii</i> , <i>Ae. speltoides</i> , and <i>T. aestivum</i> from the remaining species
	A base substitution (C to T)	62	Specific to <i>Ae. markgrafii</i>
<i>trnK</i> intron	A base substitution (A to G)	54	Separates <i>Ae. speltoides</i> and <i>T. aestivum</i> from the remaining species
	A deletion of 4 bases (ATCA)	7	Specific to <i>Ae. markgrafii</i>
	A deletion of 8 Ts	228	Specific to <i>Ae. uniaristata</i>
	A deletion of 7 Ts	228	Specific to <i>Ae. tauschii</i>
	A deletion of 6 Ts	228	Specific to <i>Ae. speltoides</i> , <i>Ae. bicornis</i> , <i>Ae. longissima</i> , <i>Ae. comosa</i> , and <i>Ae. umbellulata</i>
<i>petB&D</i>	A deletion of 5 Ts	228	Specific to <i>Ae. markgrafii</i>
	A base substitution (C to A)	2	Specific to <i>Ae. longissima</i>
	A base substitution (C to T)	77	Specific to <i>Ae. uniaristata</i>
	A base substitution (A to C)	107	Specific to <i>Ae. speltoides</i> and <i>T. aestivum</i>
	A base substitution (T to A)	448	Specific to <i>Ae. comosa</i>
	A deletion of 1 C	450	

Table 4. The matrix of similarity values generated from combined data of CAPS and sequencing.

	<i>T. aestivum</i>	<i>Ae. speltoides</i>	<i>Ae. tauschii</i>	<i>Ae. longissima</i>	<i>Ae. bicornis</i>	<i>Ae. comosa</i>	<i>Ae. uniaristata</i>	<i>Ae. umbellulata</i>	<i>Ae. markgrafii</i>	<i>A. muticum</i>
<i>T. aestivum</i>	1.00									
<i>Ae. speltoides</i>	0.90	1.00								
<i>Ae. tauschii</i>	0.64	0.59	1.00							
<i>Ae. longissima</i>	0.59	0.58	0.80	1.00						
<i>Ae. bicornis</i>	0.59	0.60	0.80	0.96	1.00					
<i>Ae. comosa</i>	0.58	0.56	0.74	0.78	0.75	1.00				
<i>Ae. uniaristata</i>	0.58	0.56	0.78	0.75	0.79	0.86	1.00			
<i>Ae. umbellulata</i>	0.62	0.60	0.85	0.86	0.86	0.79	0.80	1.00		
<i>Ae. markgrafii</i>	0.54	0.54	0.73	0.74	0.78	0.68	0.72	0.79	1.00	
<i>A. muticum</i>	0.56	0.57	0.75	0.75	0.78	0.69	0.73	0.79	0.75	1.00

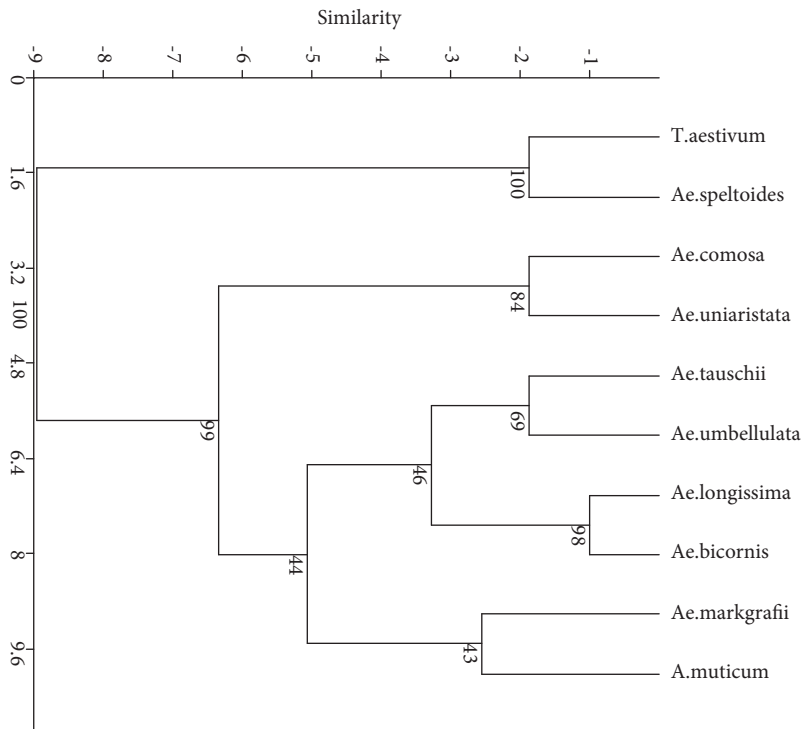


Figure 3. The phylogenetic tree of *T. aestivum* and 8 diploid *Aegilops* species. *A. muticum* is the outgroup species.

A similar scenario was observed for *Ae. tauschii* and *Ae. umbellulata* (bootstrap 69%), which differed from each other in 6 characters (5 substitutions and 1 single-base indel) for sequencing and the restriction profile generated from *trnT-L-TaqI* for CAPS. *Ae. comosa* and *Ae. uniaristata* also formed a single clade as sister species (bootstrap 84%), differing from each other only in 7 characters (5 nucleotide substitutions and 2 single-base deletions). *T. aestivum* and *Ae. speltoides* (sister species, bootstrap 100%) formed the second main cluster and differed from each other in 5 characters (3 substitutions and 2 single-base indels). There were 21 characters (substitutions and indels) supporting the idea that *T. aestivum* is a sister species to *Ae. speltoides*.

Discussion

Several studies have shown that variation in cpDNA restriction patterns can provide important clues concerning evolutionary relationships among plant species, including the *Aegilops* species (e.g. Ünlü and Sümer 2005). Therefore, Kucuk et al. (2006) amplified

the region that lies at the end of the *rbcL* gene and the beginning of the *psaI* gene in 11 wheat species of *Triticum* and *Aegilops*, restricting its amplification products with 6 restriction endonucleases. Other RFLP studies of cpDNA have provided a clear picture of the phylogeny and evolution of polyploid species of *Triticum* and *Aegilops* (Ogihara and Tsunewaki 1988). These studies revealed that bread wheat and domesticated Emmer share an identical chloroplast genome type with wild Emmer. Provan et al. (2004) utilized polymorphic chloroplast microsatellites to analyze cytoplasmic relationships between accessions in the genera *Triticum* and *Aegilops*. Results generated confirmed that *Ae. speltoides* or a closely related but now extinct species was the original B genome donor of cultivated polyploid wheat.

Yamane and Kawahara (2005) sequenced 4 noncoding regions of cpDNA to reveal interspecific phylogenetic relationships among diploid *Triticum-Aegilops* species (Poaceae). Recently, Golovnina et al. (2007) conducted a phylogenetic analysis of all known wheat species and the closely related species of *Aegilops* based on chloroplast *matK* gene comparison

along with *trnL* intron sequences of some of those species. Their results suggested that a single ancestral representative of *Ae. speltoides* participated in the speciation of polyploid wheats with a B genome in their genome composition. They also revealed strict maternal chloroplast inheritance of the synthetic wheat amphiploids included in their study. More recently, the phylogenetic tree constructed by Goncharov et al. (2008), based on few chloroplast sequences, showed that *Ae. speltoides* was a donor of the plasmon for all polyploid wheat species. Kilian et al. (2007), however, stated that inferences of the B genome progenitor are questionable (Huang et al. 2002) in the absence of genome-wide surveys for many loci. Hence, a total of 28 chloroplast loci were analyzed in this study using the restriction and sequencing of amplification products generated to investigate relationships between *T. aestivum* and 8 diploid *Aegilops* species in order to determine the chloroplast donor of *T. aestivum* and, accordingly, the B genome donor.

Compared to CAPS, sequencing revealed a higher level of polymorphism among species targeted here because it allowed the detection of differences in the loci analyzed as small as a single base. Haider (2003) reported that sequencing offers increased precision in comparison to restriction site analysis because the former permits a better homology assessment of molecular characters and character states among samples of interest.

Generally, the chloroplast noncoding regions analyzed in the current study showed a higher level of variation compared to coding regions. This is because noncoding regions evolve more rapidly than coding regions (Chiang et al. 1998). Among the 7 noncoding regions examined, *trnL-F* and the *trnK* intron displayed the highest degree of variation among the species examined. This suggests a higher rate of evolution in these regions compared to the other 5 regions. As for the coding regions analyzed, *petB&D* showed the highest level of variation compared to the remaining 20 coding regions targeted. This agrees with the observations of Haider and Wilkinson (2011), who amplified *petB&D* with the same universal primer pairs used here and compared the level of variation in this region among 96 different plant species to a number of other

coding regions of the cpDNA. Based on the results the authors obtained, they nominated *petB&D* as a suitable chloroplast region for DNA barcoding of plant species. The same region has also proved to be efficient in another study carried out by the same authors (unpublished data) for the DNA barcoding of British native grasses (Poaceae).

The little polymorphism in the cpDNA observed among the *Aegilops* species considered in this study was revealed earlier by Haider and Nabulsi (2008) based on CAPS on cpDNA. Langridge et al. (2001) also reported the low level of polymorphism in wheat relative to other cereals.

Identification of the B genome donor of bread wheat

There are a number of published reports about the origin of the B genome in tetraploid and hexaploid wheats (e.g. Baum et al. 2009). However, the donor of the B genome is still controversial and remains uncertain (Golovnina et al. 2007).

The ambiguity and conflicting results in most of the methods employed in deducing the precise B genome donor(s) of bread wheat can be attributed to the special evolutionary characteristics of the particular portion of the genome analyzed in nominated progenitor species (Harlan 1992). This may also be due to the fact that each type of experiment can only provide limited information. For instance, proteins and isozymes have only a few loci available for analysis (Haider et al. 2010). Furthermore, inadequate sampling of the level of variation within a putative diploid donor species can yield conflicting results when phylogenetic relationships for a polyploid genome are studied. Chromosome morphology and banding cannot be accurate, for example, because chromosome morphology may be changed by rearrangements, and there is usually extensive C-banding polymorphism, not only between different species but also within a given species (Fernandez-Calvin and Orellana 1990).

A second explanation for the ambiguity is that the B genome donor may either be extinct (Talbert et al. 1991) or the B genome itself may have become modified at the tetraploid level (Sarkar and Stebbins 1956). Talbert et al. (1991), however, suggested *Ae. speltoides* as the closest living species to the extant

species. Zhang et al. (2008) also pointed out that the B genome donor is believed to be extinct, heavily modified, or not yet discovered, but agreed that it was probably an ancestor of *Ae. speltooides*.

Because different types of analysis have implicated different species as the B genome donor of polyploid wheat (Blake et al. 1999), an alternative explanation would be that the B genome is polyphyletic in origin, that it is a recombined genome derived from 2 or more diploid *Aegilops* species (Liu et al. 2003). Such a polyphyletic origin would result in a high level of differentiation in the B genome (Harlan 1992), and may arise from mixed genomes in which genetic interchanges occur between tetraploid AABB amphidiploids carrying different putative B genome donor species but a common A genome.

A polyphyletic origin of the B genome was also suggested based on karyotype analysis (Giorgi and Bozzini 1969), DNA satellite distribution (Dennis et al. 1980), enzyme analysis (Nishikawa et al. 1992), chromosome pairing (Joppa 1993), chromosome banding patterns (Natarajan and Sarma 1974), and a low-copy noncoding chromosome-specific DNA sequence (Liu et al. 2003). Blake et al. (1999), however, supported the monophyly of the B genome of wheats. Talbert et al. (1995) came to the same conclusion based on sequences of low-copy DNA from the B genome in bread wheat and its putative diploid donors. In order to elucidate this problem, Golovnina et al. (2007) conducted a phylogenetic analysis of all known wheat species and the closely related species of *Aegilops* based on the sequence comparison of the chloroplast *matK* gene with the *trnL* intron of some species. The S genome of *Ae. speltooides* appeared to be most closely related to the B genome. Provan et al. (2004) reported that the progenitor is now widely assumed to have been a member of section *Sitopsis* of genus *Aegilops*, such as *Ae. bicornis*, *Ae. longissima*, or, most probably, *Ae. speltooides*.

Ae. speltooides was the first species of the section to be considered closely related to the B genome donor of bread wheat or as the putative donor based on morphological data (Sarkar and Stebbins 1956), karyotype data (Riley et al. 1958), chromosome banding (Chen and Gill 1983), the amount of nDNA (Pegington and Rees 1970), plasmon analysis (Tsunewaki 1991), glutenin (important components

of seed storage proteins) SDS-electrophoresis (Fernandez-Calvin and Orellana 1990), *rbcL* sequences (Terachi et al. 1988), isoenzyme data (Jaaska 1978), C-banding of chromosomes (Friebe and Gill 1996), cytology (Kerby and Kuspira 1988), electrophoretic mobilities of proteins (Johnson 1972), the geographical distributions of wild wheat populations (Witcombe 1983), RFLP analysis of low-copy DNA sequences (Talbert et al. 1995), highly repetitive DNA sequences and ribosomal RNA (Badaeva et al. 1996a, 1996b), meiotic chromosome pairing (Maestra and Naranjo 1998), and 5S rDNA units (Baum et al. 2009). *Ae. speltooides* was even proposed as the mitochondrial genome donor of polyploid wheats (Wang et al. 2000). Added to the large body of molecular-based evidence that nominates *Ae. speltooides* as the B genome donor of cultivated wheats, morphological, geographical, and cytological evidence also suggest that *Ae. speltooides* (S genome) or a closely related species was the B genome ancestor (van Herpen et al. 2006).

In this study, the phylogenetic tree revealed *Ae. speltooides* to be distinct from the remaining 2 *Sitopsis* species and all remaining *Aegilops*. Haider and Nabulsi (2008) and Kawahara et al. (2008) reached the same conclusion based on CAPS and sequencing of coding and noncoding regions of cpDNA. This was also reported earlier by the classifications of Eig (1929) and based on electrophoresis of seedling proteins (Bahrman et al. 1988), cpDNA and mtDNA (Terachi et al. 1990; Wang et al. 1997), nRFLP (Sasanuma et al. 1996), rRNA (Jiang and Gill 1994), repetitive DNA sequences (Taberlet et al. 1991), isozymes (Jaaska 1978), and sequences of nrITS (Zhang et al. 2002). The tree also showed that the only *Aegilops* species that was included in the *T. aestivum* group was *Ae. speltooides*. This observation is compatible with other studies that have revealed identical cpDNA restriction patterns between *Ae. speltooides* and bread wheat (Ogihara and Tsunewaki 1988; Haider and Nabulsi 2008). This was also in agreement with chloroplast *rbcL* analysis (Chen et al. 1975) and analysis of 3 cytoplasmically encoded proteins (Bahrman et al. 1988).

In contrast to the few previous studies that argued against *Ae. speltooides* being the B genome donor (e.g. Maestra and Naranjo 1998), the present study revealed

that *Ae. speltoides* is the diploid *Aegilops* species most likely to be the donor (Kilian et al. 2007) or the species most closely related to the ancestor of the B genome, as was proposed earlier based on microsatellites (Sourdille et al. 2001), the internal transcribed spacer sequences of nrDNA (Gulbitti-Onarici et al. 2009), and comparative analysis of nucleotide sequences in rDNA promoter regions (Vakhitov et al. 2003). Uncertainty remains, however, regarding whether *Ae. speltoides* is the sole source of the B genome or whether this genome resulted from an introgression of several parental species (Blake et al. 1999).

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