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The classification of tetraploid wheat by phylogenetic and cytogenetic analyses

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Abstract

Tetraploid wheat (*Triticum turgidum* L.) is an important species within the genus *Triticum* and harbors many desirable agronomic traits. The classification, origin, and evolution of tetraploid wheat remain confused and controversial, resulting in useless germplasm resources. Two classification systems for tetraploid wheat are widely used: *1*) tetraploid wheat comprises two species; *2*) all forms of tetraploid wheat are classified as one species. The present study aimed to reassess the classification of tetraploid wheat using phylogenetic analysis of nuclear rDNA internal transcribed spacer region (ITS) sequence data, fluorescence *in situ* hybridization (FISH) karyotyping, and observation of meiotic pairing behavior in F_1 hybrids. Network analysis of ITS sequences indicates that tetraploid wheat was not closely related to other Triticeae species with the exception of *Aegilops speltoides* and *Ae. sharonensis*. Phylogenetic analysis of ITS sequences and FISH show that *Triticum turgidum* and *T. timopheevii* clustered on distinct branches, and meiotic pairing in F_1 hybrids of these species showed a high frequency of univalents. Meiotic behavior of F_1 hybrids among forms of *T. turgidum* revealed a low number of univalents (means < 2) except for *T. turgidum* ssp. *dicoccoides*. The significant variation on chromosomes 1A, 2A, 5A, 1B, 2B, 3B, and 6B in the FISH hybridization patterns were observed between *T. turgidum* ssp. *dicoccoides* and other *T. turgidum* accessions. Furthermore, the results of ITS phylogenetic analyses correspond closely with observations of meiotic behavior and FISH karyotyping. The present results indicate that *T. turgidum* and *T. timopheevii* are two distantly related species of different origins. *Triticum turgidum* ssp. *dicoccoides* should be maintained as a subspecies of *T. turgidum* whereas other forms of *T. turgidum* should be reclassified as varieties.

Additional key words: chromosome pairing behavior, FISH analysis, ITS, *Triticum turgidum*.

Introduction

Tetraploid wheat (*Triticum turgidum* L.) has played an important role in the history of human civilization. As relatives of *Triticum aestivum* L., tetraploid wheat possesses many desirable agronomic traits and thus can be used as parents in breeding programs to improve the yield and quality of *T. aestivum* (Zaharieva *et al*. 2010). With hard kernel texture and abundant protein for pasta, *T. turgidum* ssp. *durum* has become the second cultivated wheat after *T. aestivum* (Oliveira *et al*. 2012). *Triticum turgidum* ssp. *durum* was crossed with *Thinopyrum elongatum* (Host) A. Löve, and a series of addition lines and substitution lines were successfully raised as breeding material to enhance the Fusarium head blight resistance of wheat cultivars (Jauhar *et al*. 2008, 2009, 2014, Forte *et al*. 2014, Kuzmanović *et al*. 2014). Many valuable genes have been identified in *T. turgidum* ssp. *dicoccoides* (Körn. *ex* Aschers. *et* Graebn. Thell.), including the stripe rust resistance genes *Yr15* and *YrH52*, the powdery mildew resistance genes *Pm16*, *Pm26*, and *Pm30*, and the high grain protein content gene *GPC-B1*, which are widely used in breeding for high yield (Gerechmr-Amitai *et al*. 1989, Reader and Miller 1991, Peng *et al*. 1999, Rong *et al*.

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Abbreviations: AFLP - amplified fragment length polymorphism; DAPI - 4-6-diamino-2-phenylindole; FISH - fluorescence *in situ* hybridization; ITS - internal transcribed spacer region; MJ - median-joining; NJ - neighbor-joining; PMC - pollen mother cell.
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2000, Liu *et al*. 2002, Chen *et al*. 2005, Uauy *et al*. 2006, Peleg *et al*. 2008). Other forms of tetraploid wheat are also excellent genetic resources for precocity, pest resistance, lodging resistance, salt tolerance, or nutrient abundance (Oliveira *et al*. 2012).

For effective use of germplasm resources, a strictly defined classification of tetraploid wheat is crucial (Goncharov *et al.* 2009). Taxonomists have proposed different classification systems based on morphological, cytological, or genetic characteristics, and consequently the determination of species and subspecies of tetraploid wheat is extremely confusing and controversial (Goncharov *et al*. 2011). Bowden (1959) treated allotetraploid wheat as one species, *T. turgidum*, with three varieties, two variants, and eight cultivars. Morris and Sears (1967) recognized *T. timopheevii* as a species based on cytogenetic evidence and geographic distribution. Two major classification systems for tetraploid wheat have been proposed: *1*) MacKey (1966) and Van Slageren (1994) classified all tetraploid wheat as subspecies, MacKey (1975) further classified *durum* and *turanicum* ssp. as cultivars of *T. turgidum*; *2*) on the basis of morphological observations, Dorofeev *et al*. (1979) concluded that all ssp. of tetraploid wheat are individual species.

Given genetic and morphological diversity, the evolution of tetraploid wheat under domestication has not been widely reported (Matsuoka *et al.* 2011). Domesticated *Triticum turgidum* (emmer wheat) is suggested to have originated from *T. turgidum* ssp. *dicoccoides* in southeastern Turkey (Özkan *et al*. 2011). Genetic and archaeological evidence indicate that following the initial domestication of *T. turgidum* ssp. *dicoccoides*, naked tetraploid wheats evolved from emmer wheat (Salamini *et al*. 2002, Özkan *et al*. 2005). Using cluster analysis of amplified fragment length polymorphism (AFLP), allele frequencies revealed that all hulled wheats are clustered in one group, and all types of naked wheat are included in a distinct group (Salamini *et al*. 2002). *Q* gene sequences indicate that tetraploid wheat can be divided into two clades supported by spike morphological traits, namely non-free-threshing, fragile, and free-threshing, non-fragile, normal wheats (Sormacheva *et al*. 2015). On the basis of genetic diversity indicated by four sets of genetic markers, Oliveira *et al*. (2012) emphasized that *T. turgidum* ssp. *durum* and *T. turgidum* ssp. *turgidum* share a common gene pool and comprise a genetically different population from *T. turgidum* ssp. *dicoccon* and *T. turgidum* ssp. *dicoccoides.* Phylogenetic analysis of four single-copy nuclear gene showed that each clade contained naked tetraploid wheat and hulled tetraploid wheat, which is indicative of a close evolutionary relationship between these two forms of tetraploid wheat (Takenaka *et al*. 2010). Haplotype analysis of sequence data for the photoperiod-related *Ppd-A1* and *Ppd-B1* genes in tetraploid wheat indicated that gene flow between hulled and naked tetraploid wheat had occurred (Takenaka *et al*. 2012). Phylogenetic reconstruction of *Ppd-A1b* haplotypes revealed that *T. turgidum* ssp. *turgidum* and *T. turgidum s*sp. *ispahanicum* were not clustered together with *T. turgidum* ssp. *dicoccoides*, *T. turgidum* ssp. *durum*, *T. turgidum* ssp. *dicoccon,* and *T. turgidum* ssp. *carthlicum* were closely related (Muterko *et al*. 2015). Thus, despite considerable research, the phylogenetic history of tetraploid wheat remains contentious.

Our previous study indicated that the hulled tetraploid wheat accessions formed a subclade, and naked tetraploid wheat got other subclade, and at least two intermediary subspecies were involved in the evolution of *T. turgidum* (Tang *et al*. 2017). In this study, we further sequenced and analyzed the nuclear rDNA internal transcribed spacer region (ITS) sequences, observed chromosome pairing in artificial F_1 hybrids, and assessed the chromosomal distribution of signal sites for the fluorescence *in situ* hybridization (FISH) probes pTa535 and pSc119.2 to re-evaluate the phylogenetic relationships and taxonomic classification of tetraploid wheat.

Materials and methods

Plant materials: Eleven taxa of tetraploid wheat were used for the ITS sequence analysis. The genome composition, provenance, and *GenBank* accession numbers for samples included in the study are listed in Table 1 Suppl. The ITS sequences with MH numbers of tetraploid wheat are newly reported in this study. The sequences for related species were downloaded from the *GenBank* database. *Bromus inermis* was used as outgroup. A collection of 21 tetraploid wheat accessions including 11 taxa were employed for FISH (Table 1 Suppl.). The F_1 hybrids derived from 14 artificial crosses between tetraploid wheat accessions were used to study meiotic pairing behavior (Table 1). The accessions designated by PI and Citr numbers were kindly provided by *American National Plant Germplasm System* (Pullman, USA), and the accessions with AS numbers were collected by Triticeae Research Institute, Sichuan Agricultural University, China.

Phylogenetic analysis: Total genomic DNA was extracted from fresh leaf tissue using the standard cetyltrimethylammonium bromide extraction protocol (Doyle and Doyle 1990). Primers ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-L (5′-TCGTAACA AGGTTTCCGTAGGTG-3′) were used to amplify the entire ITS region (Hsiao *et al*. 1995). The PCR reaction was accomplished in a 50 mm^3 volume containing 5 mm^3 of 10× *Taq* polymerase buffer, 1.5 mM of each primer, 10 mM of dNTP mix, 20 ng of template DNA, 2.5 mM $MgCl_2$, and 2 U high-fidelity *ExTaq*® DNA polymerase (*Takara*, Dalian, China). A *GeneAmp*® *9700* thermal cycler (*Applied Biosystems*, Foster City, CA, USA) was used to perform the PCR reaction. The amplification procedure consisted of pre-denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, at 52 °C for 1 min, and at 72 °C for 1 min, followed by 72 °C for 7 min. The PCR products were cloned into the pMD19-T vector (*Takara*) by TA cloning. For recovery of all possible ITS sequences, 10 clones per accession were randomly selected to sequence in a single direction by the *TSINGKE Company* (Beijing, China). The sequences were aligned

online against the *National Center for Biotechnology Information* database to confirm the accuracy.

Multiple sequence alignments were generated using *ClustalX* software and manually adjusted using *MEGA 5.0* software (Thompson *et al*. 1999, Tamura *et al*. 2011). In the initial phylogenetic analysis, only one sequence was retained when multiple sequences from the same accession formed a monophyletic group. Basic sequence data, including nucleotide frequencies, the transition/transversion ratio, and variability, were recorded with *MEGA* software. *DnaSP 4.0* was employed to calculate the number of polymorphic sites, total number of mutations, number of haplotypes, haplotype diversity, and nucleotide diversity (Rozas *et al*. 2003).

Three sequence matrices (I, II, and III) were used to examine phylogenetic relationships, and putative relative species were included in each data set. Matrix I comprised all ITS sequences for tetraploid and relative species in *Triticeae*, and was subjected to phylogenetic network analysis with *SplitsTree 4* using the *NeighborNet* algorithm (Bryant and Moulton 2004, Huson and Bryant 2005). To clarify the relationship between tetraploid wheat and *Aegilops*, matrix II, including one copy sequence of tetraploid wheat in a monophyletic group, *Aegilops*, and the putative A genome donor, was analyzed with the neighbor-joining (NJ) algorithm using *MEGA 5.0*. A bootstrap analysis with 1 000 replicates was performed to statistical support for the NJ tree topology. To further explore the evolution of ITS sequences in tetraploid wheat, matrix III, which comprised the ITS sequences for all accessions of tetraploid wheat, was subjected to a median-joining (MJ) network analysis using *Network v5.0* software (Allaby and Brown 2001).

Fluorescence in situ hybridization analysis: In this study, we used pSc119.2 and pTa535 as probes. The probe pSc119.2 (6-FAM-5′CCGTTTTGTGGACTA TTACTCACCGCTTTGGGGTCCCATAGCTAT3′) from rye repetitive sequences was used to determine the B genome chromosomes of wheat. The probe pTa535 (Tamra-5′AAAAACTTGACGCACGTCACGTACA A A T T G G A C A A A C T C T T T C G G A G T A T C A GGGTTTC3′) from wheat repetitive sequences hybridizes preferentially to A and D genome chromosomes (Tang *et al*. 2014). Thus, binding of the two probes can be used to identify the wheat A and B chromosomes. Both probes were synthesized by *Invitrogen* (Shanghai, China). The FISH procedure followed the method of Han *et al*. (2006), with slight modifications. The probe mixture (0.35 mm^3 of each probe in 2× SSC and 1× TRIS-EDTA buffer, pH 7.0, total volume 10 mm^3) was dropped on a slide, covered with a coverslip, stored in a moist box at 37 °C for 2 h and washed in 2× SSC at room temperature. Chromosomes were counterstained with DAPI (4-6-diamino-2-phenylindole) solution (*Vector Laboratories*, Burlingame, CA, USA). Photomicrographs of FISH chromosomes were taken with an *Olympus BX-51* (Tokyo, Japan) microscope equipped with a *DP-70 CCD* camera and all images were processed with *Photoshop CS 5.0* (*Adobe Systems*, San Jose, CA, USA). *Excel* software was used to count the number of signal sites, and *SLT_NTsys2.1e* software was used to normalize the matrix of signal sites, calculating coefficient of genetic similarity and neighbor-joining (NJ) algorithm to create a cluster analysis (Mantel 1967).

Meiotic pairing analysis: The F_1 hybrids included in this study are listed in Table 1. Stages of meiosis were determined in acetocarmine squashes of one of three anthers per flower. At the appropriate stage, the remaining two anthers were fixed in a mixture of absolute ethanol, chloroform, and acetic acid (6:3:1, v/v/v) kept in a refrigerator for 24 h, then stored at 4 °C in 70 % (v/v) alcohol. At least 30 pollen mother cells (PMCs) per plant were identified.

Results

A total of 70 ITS sequences for tetraploid wheat accessions were obtained by PCR amplification, cloning, and sequencing. For *T. turgidum* ssp. *carthlicum* accession PI532494 ITS copies were obtained from the A genome only, whereas for the remainder of the tetraploid wheat accessions ITS sequences were obtained from the B or G genomes. The amplified ITS sequence consisted of the ITS1 region, 5.8S rRNA gene, and ITS2 region. The total length of the sequence was 594 - 608 bp, of which the ITS1 region was 216 - 218 bp, the ITS2 region was 214 - 216 bp, and the 5.8S gene was 164 - 165 bp. The number of loci was 606, including 330 conserved characters, 268 variable characters, and 146 parsimony informative characters. The four residue frequencies were A = 21.5 %, T/U = 17.3 %, G = 28.3 %, and C = 32.9 %, respectively. The transition/transversion ratio was 2.66. In the multiple sequence alignment generated with *ClustalW*, no indel was detected among the tetraploid wheat ITS sequences. The number of haplotypes (h) among the ITS sequences was 101, and the haplotype (gene) diversity (Hd) was 0.989. The nucleotide diversity (Pi) value of 0.03672 indicated that the tetraploid wheat ITS sequences showed high genetic diversity.

To explore the taxonomic status of tetraploid wheat in the Triticeae, a network analysis including relative species was performed using *SplitsTree 4.0*. The network showed that the ITS sequences were split into two major clades (Fig. 1 Suppl.). Clade I originated from non-B and non-G genome types, which included all of the relative species with the exception of *Aegilops* species. Tetraploid wheat, as expected, was clustered with *Aegilops* species, and hexaploid wheat was included in Clade II. The Clade II comprised two subclades, which consisted of sequences from the B and G genomes. Subclade I consisted of the sequences from *T. timopheevii and Aegilops* species. In Subclade II, one, two, or three copies of ITS sequences were obtained from the accessions of *T. turgidum*.

To further examine the phylogenetic relationships between tetraploid wheat and the diploid donor species, all of the tetraploid wheat ITS sequences were included in a NJ analysis, together with five putative donors of *Aegilops* in the Triticeae (Fig. 1). Only one sequence was retained in the data set when the same accession was clustered in

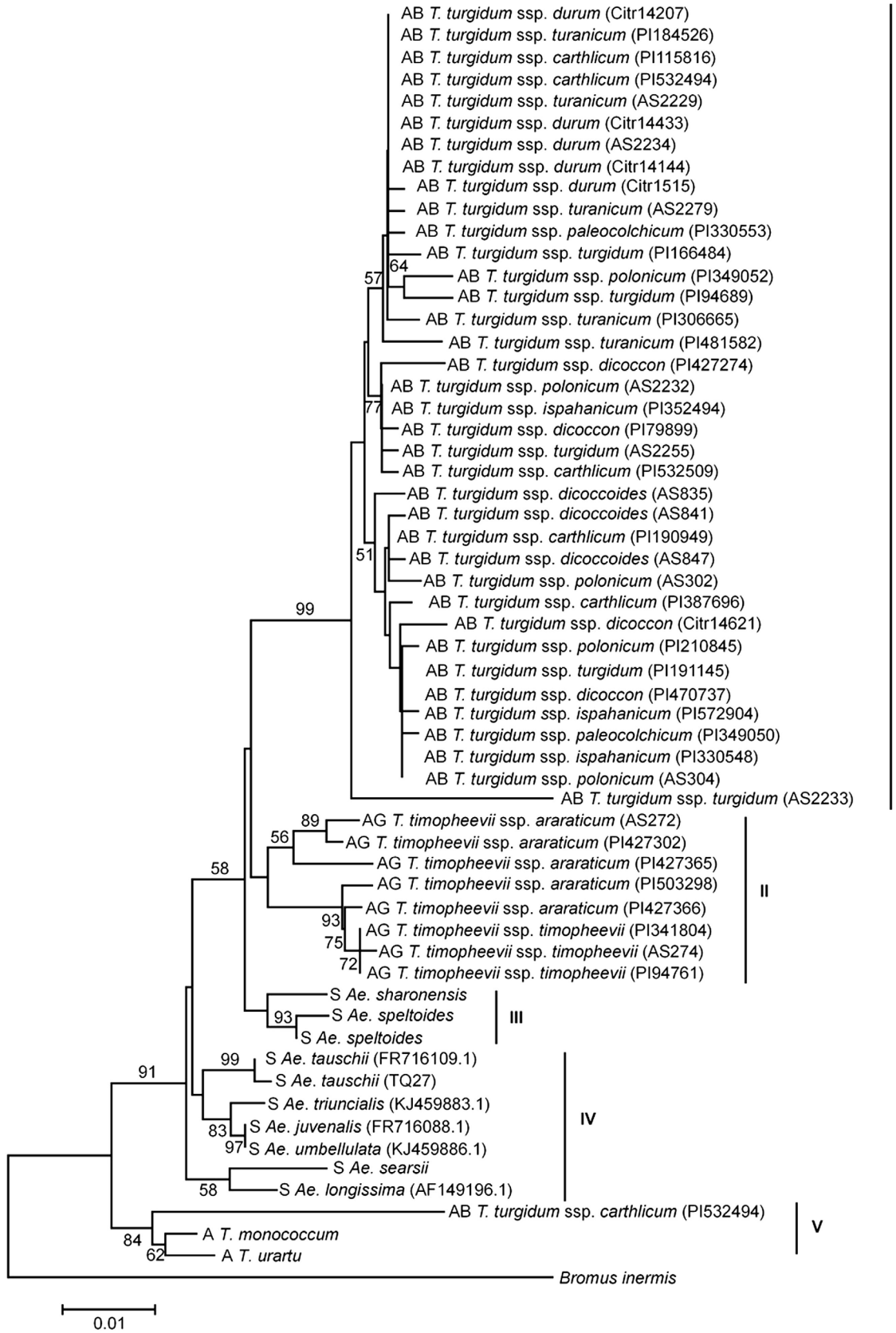

Fig. 1. A neighbor-joining (NJ) tree derived from internal transcribed spacer region (ITS) sequences of tetraploid wheat and putative diploid donor species. The NJ tree divides all the ITS sequences into five clades. The species name and accession number are indicated for each taxon. Bootstrap support values greater than 50 % are shown above the branches.

a monophyletic group. Five major clades were resolved. In Clade I, all *T. turgidum* sequences were grouped with high bootstrap support (99 %), representing the B genome sequences. Clade I have three subclades with the varieties of *T. turgidum* separately, Clade II included all *T. timopheevii* sequences with the G genome sequences, Clade III consisted of two *Ae. speltoides* accessions and one *Ae. sharonensis* accession. The remainder of the *Aegilops* species formed Clade IV. *Triticum turgidum* ssp. *carthlicum* PI532494 was grouped with *T. monococcum* and *T. urartu* in Clade V. To highlight the relationships among haplotypes of the ITS sequence, a network analytical method was used. A MJ analysis of the haplotype data showed the relationship between *T. timopheevii* and *T. turgidum* (Fig. 2), which corresponded to clades revealed in the NJ phylogeny. All haplotypes derived from *T. turgidum* or *T. timopheevii* were clustered together, respectively. The MJ analysis further divided *T. turgidum* into major four parts. All *T. turgidum* ssp. *turanicum* and *T. turgidum* ssp. *durum* sequences were included in subgroup I. *Triticum turgidum* ssp. *dicoccon* (PI79899 and PI427274), *T. turgidum* ssp. *polonicum* (AS2232), *T. turgidum* ssp. *ispahanicum* (PI 352494), *T. turgidum* ssp. *turgidum* (AS2255), *and T. turgidum* ssp. *carthlicum* (PI532509) formed subgroup II. Most accessions of *T. turgidum* ssp. *dicoccoides* were included in subgroup III. Subgroup IV consisted of five haplotypes, including *T. turgidum* ssp. *dicoccon* (PI470737 and Citr14621), *T. turgidum* ssp. *polonicum* (AS304 and PI210845), *T. turgidum* ssp. *turgidum* (PI191145), *T. turgidum* ssp. *paleocolchicum* (PI349050), and *T. turgidum* ssp. *ispahanicum* (PI572904).

To further explore phylogenetic relationships and genetic diversity of tetraploid wheat, chromosomal differentiation in tetraploid wheat accessions was analyzed by FISH using probes that targeted chromosomal sites mainly in the A, B, and G genomes.

On the A genome, the probes pSc119.2 and pTa535 produced abundant signals, and many differences in signals were observed on chromosomes 1A, 2A, 4A, and 5A among *T. turgidum* and *T. timopheevii* accessions (Fig. 3). The pSc119.2 signals were only observed on chromosome 1AS, 1AL, 2AS, 2AL, 4AL, 5AS, and 5AL (S and L represent the short and long arm of the chromosome, respectively), and hybridization patterns were variable. The terminal pSc119.2 signals on chromosome 1AS was only detected in five accessions, including *T. turgidum* ssp. *dicoccoides* (AS841 and PI470947), *T. turgidum* ssp. *ispahanicum* (PI330548), *T. turgidum ssp. paleocolchicum* (PI349050), and *T. turgidum* ssp. *carthlicum* (PI387696) accessions. Two accessions of *T. timopheevii* ssp. *timopheevii* were identified by strong subterminal signals of pSc119.2 on chromosome 1AL. This probe hybridized to the terminal region of chromosome 2AS only in *T. turgidum* ssp. *turgidum* (AS2255). The strong terminal

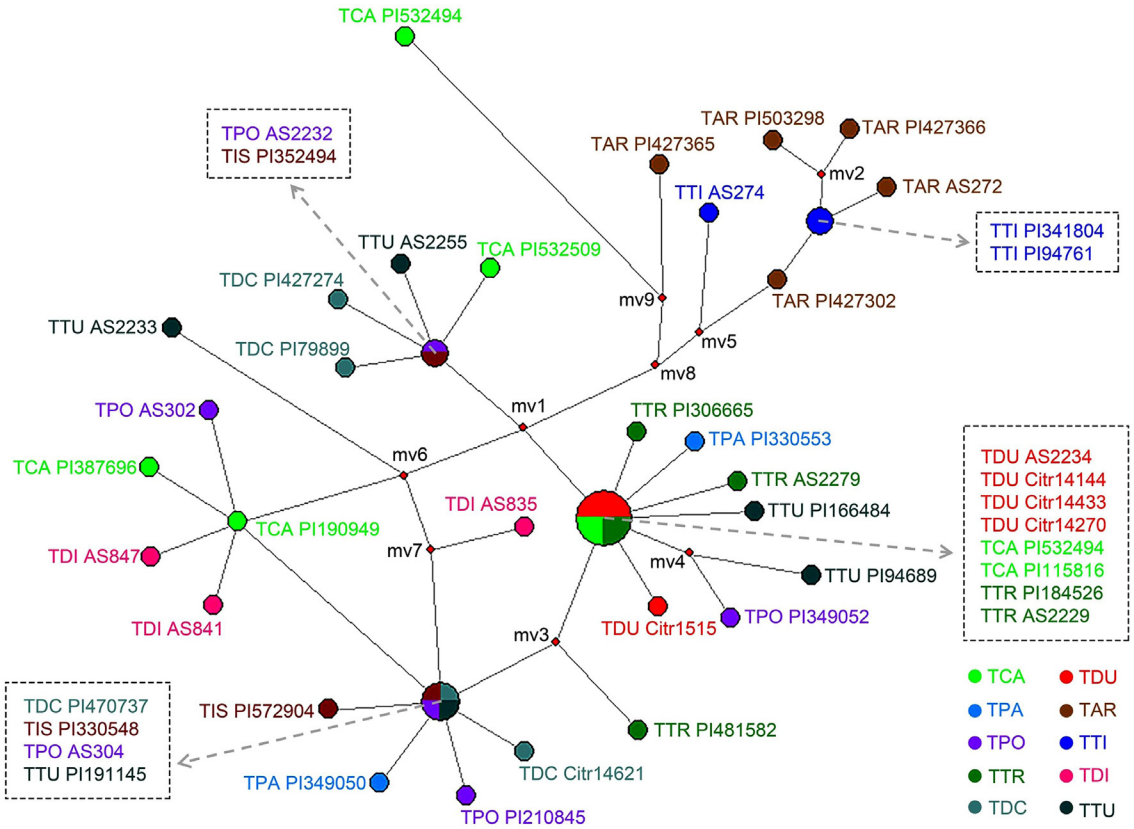

Fig. 2. A median-joining network derived from internal transcribed spacer region sequences of tetraploid wheat. Abbreviations for the species are listed in Table 1 Suppl. Each *circle* represents a haplotype, each *color* represents one species, and the node size is proportional to the number of haplotypes. Median vectors (mv) represent inferred unsampled nodes. Numbers on branches indicate a mutation site.

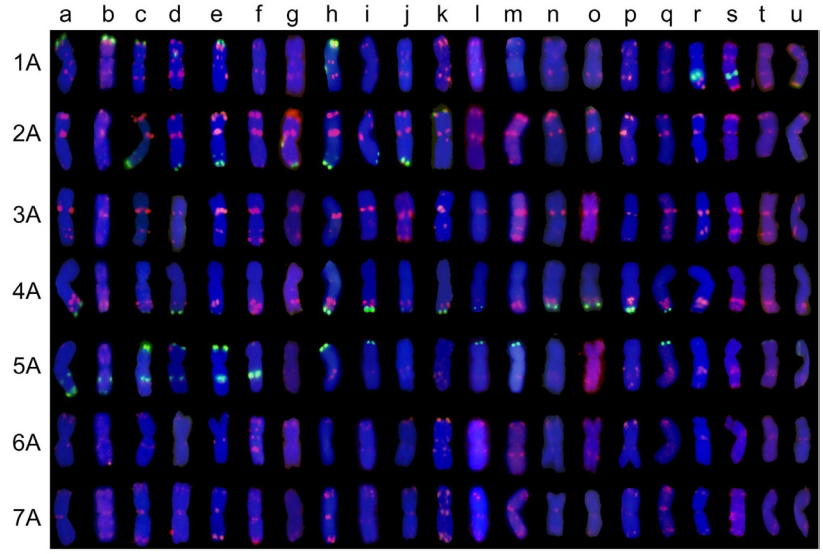

Fig. 3. Fluorescence *in situ* hybridization probe signals on genome chromosomes A of tetraploid wheat for pSc119.2 (*green*) and pTa535 (*red*). Columns a to u - *Triticum turgidum* ssp. *dicoccoides* (PI470947), *T. turgidum* ssp. *dicoccoides* (AS841), *T. turgidum* ssp. *ispahanicum* (PI330548), *T. turgidum* ssp. *ispahanicum* (PI572904), *T. turgidum* ssp. *paleocolchicum* (PI349050), *T. turgidum* ssp. *dicoccon* (Citr3686), *T. turgidum* ssp. *dicoccon* (PI427274), *T. turgidum* ssp. *carthlicum* (PI387696), *T. turgidum* ssp. *carthlicum* (PI115816), *T. turgidum* ssp. *turgidum* (PI191145), *T. turgidum* ssp. *turgidum* (AS2255), *T. turgidum* ssp. *polonicum* (PI366117), *T. turgidum* ssp. *polonicum* (AS304), *T. turgidum* ssp. *turanicum* (PI481582), *T. turgidum* ssp. *turanicum* (AS2229), *T. turgidum* ssp. *durum* (Citr14144), *T. turgidum* ssp. *durum* (Citr14433), *T. timopheevii* ssp. *timopheevii* (PI282933), *T. timopheevii* ssp. *timopheevii* (Citr15205), *T. timopheevii* ssp. *araraticum* (PI427366), and *T. timopheevii* ssp. *araraticum* (AS272).

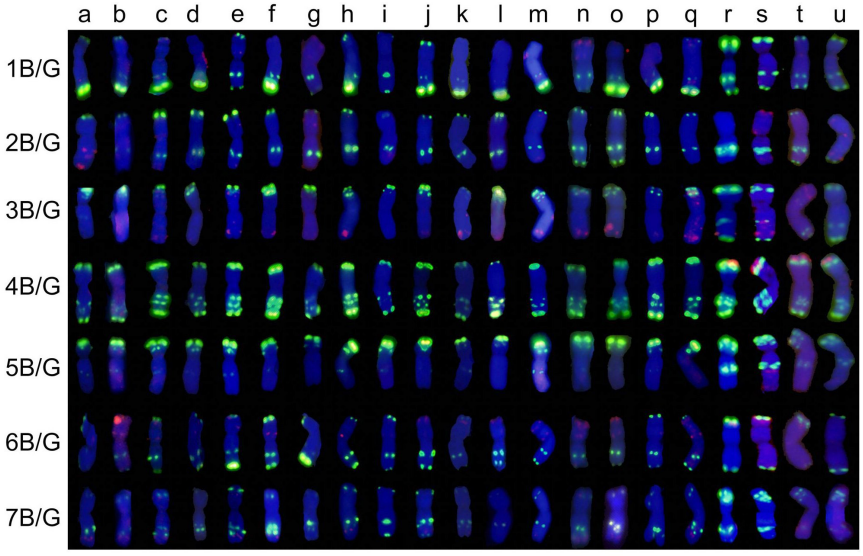

Fig. 4. Fluorescence *in situ* hybridization probe signals on genome chromosomes B and D of tetraploid wheat for pSc119.2 (*green*) and pTa535 (*red*). The accession numbers are the same as those listed in Fig. 3.

pSc119.2 signals on chromosome 2AL were found among seven accessions. The signal located in the terminal region of 4AL was observed in almost all accessions, excluding *T. turgidum* ssp. *dicoccon (*Citr3686), *T. turgidum* ssp. *polonicum* (AS304), *T. timopheevii* ssp. *timopheevii,* and *T. timopheevii* ssp. *araraticum.* The hybridization patterns on chromosome 5AL of *T. turgidum* ssp. *dicoccoides* (AS841 and PI470947) differed from other accessions with clear terminal and subterminal signals. *T. turgidum* ssp. *ispahanicum* (PI330548 and PI572904) also showed strong signals on the terminal region of chromosome 5AS and subterminal region of 5AL, respectively. Signal sites for the pTa535 probe were completely present on 1A-7A chromosomes of all the accessions. We found that the pTa535 site diversities on chromosome 1A, 2A, and 7A were low. In the terminal region of chromosome 3AL, only *T. turgidum* ssp. *paleocolchicum* (PI349050), *T. turgidum* ssp. *carthlicum* (PI387696), *T. turgidum* ssp. *carthlicum* (PI115816), *T. turgidum* ssp. *polonicum* (PI366117), and *T. turgidum* ssp. *turanicum* (PI481582) lacked red pTa535

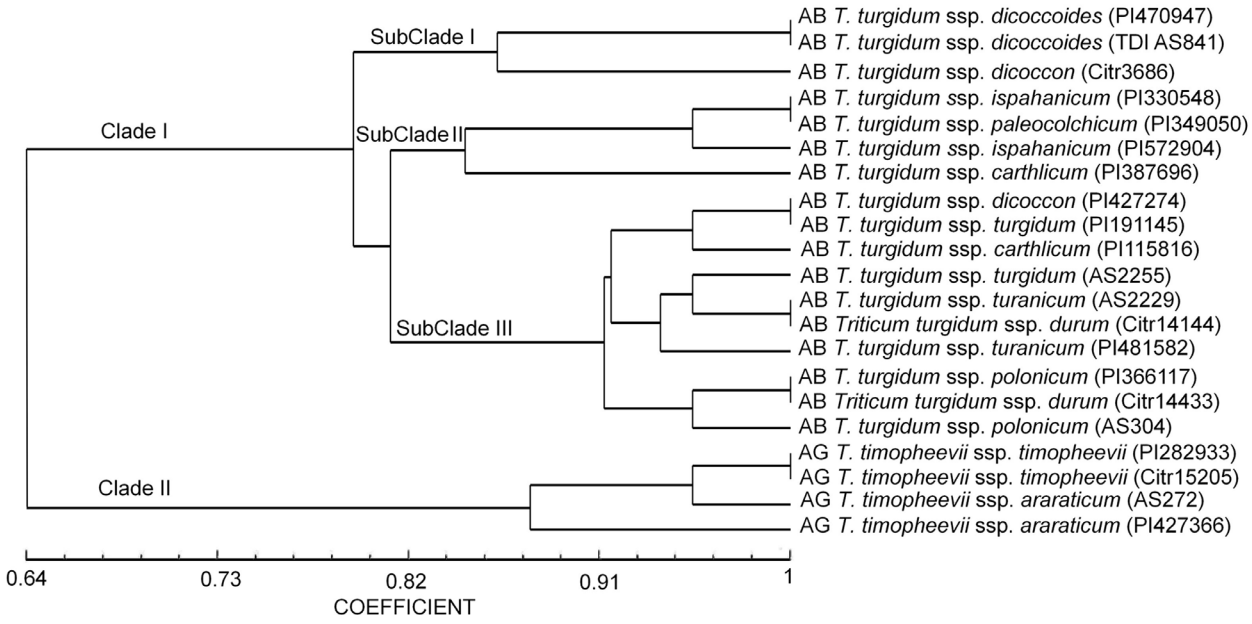

Fig. 5. A neighbor-joining tree derived from fluorescence *in situ* hybridization signal sites in tetraploid wheat.

signals. The probe pTa535 gave strong signals on the terminal and the terminal-subterminal regions on the long arm of chromosome 4A, respectively. The signal sites of pTa535 on chromosome 5AL was present in all accessions except for *T. turgidum* ssp. *dicoccoides* (PI470947), *T. turgidum* ssp. *dicoccoides* (AS841), *T. turgidum* ssp. *ispahanicum* (PI330548), *T. turgidum* ssp. *ispahanicum* (PI572904), *T. turgidum* ssp. *paleocolchicum* (PI349050), and *T. turgidum* ssp. *dicoccon* (Citr3686). Furthermore, only *T. turgidum* ssp. *carthlicum* (PI387696) lacked the faint signals near the centromere on chromosome 6AL.

Signal sites for the probe pSc119.2 were observed on all B-genome chromosomes of all accessions (Fig. 4). Hybridization differences for this probe were observed on all chromosomes except 3B and 4B. Three pairs of signals were detected on chromosome 1B for all accessions except *T. turgidum* ssp. *ispahanicum* (PI330548), *T. turgidum* ssp. *dicoccon* (Citr3686 and PI427274), *T. turgidum* ssp. *polonicum* (AS304 and PI366117), *T. turgidum* ssp. *turanicum* (PI481582 and AS2229), and *T. turgidum* ssp. *durum* (AS2234). The most characteristic signal patterns were generated in two accessions *T. turgidum* ssp. *turanicum* with two pairs of signals on chromosome 2BL and one pair of signals on chromosome 2BS. However, only terminal pTa119.2 signals were detected on chromosome 2BS of the accessions *T. turgidum* ssp. *dicoccoides*. On chromosome 6B, different signal combinations were observed among 11 taxa. Three pairs of signals on chromosome 7B were only observed in *T. turgidum* ssp. *turgidum (*PI191145) and *T. turgidum* ssp. *carthlicum* (PI115816). Signal sites for pTa535 were mainly apparent in the terminal region of chromosomes 3BL and 6BS, and showed less diversity. Chromosome 2BL of *T. turgidum* ssp. *dicoccoides* carried terminal and subterminal pTa535 signals, which differed from other accessions.

We found that all G-genome chromosomes could be unambiguously identified using a combination of pSc119.2 and pTa535 repeats, and the G-genome chromosomes of *T. timopheevii* and B-genome chromosomes of *T. turgidum* exhibited significant variation on all seven homologous chromosomes in the probe hybridization patterns (Fig. 4). Chromosome 1G carried strong terminal pSc119.2 signals on both arms, and strong pSc119.2 signals were found on the subterminal region of the long arm. Chromosome 2G of *T. timopheevii* ssp. *timopheevii* was characterized by strong subterminal signals of pSc119.2 on long arm and faint terminal pTa535 signals on the short arm, which differed from those of *T. timopheevii* ssp. *araraticum*. Clear terminal pSc119.2 signals, subterminal pTa535 signals, and faint pSc119.2 signals near the centromere were detected on the long arm of chromosome 3G, and the short arm produced strong telomeric pSc119.2 signals. Strong terminal and two subterminal pSc119.2 signals were detected on the long arm of chromosome 4G, and interestingly, the short arm simultaneously produced strong telomeric pSc119.2 and pTa535 signals. Compared to B-genome chromosomes, chromosome 5G had strong pSc119.2 signals near the centromere region, chromosome 6G lacked specific pSc119.2 signals subterminally on the long arm, and chromosome 7G showed strong pSc119.2 signals on the subterminal region of the short arm.

Given that copies of ITS sequences for tetraploid wheat were only obtained from the B or G genomes, cluster analysis of FISH signal site statistics located in the A genome was performed using the NJ method (Fig. 5). As expected, the 17 accessions of *T. turgidum* and 4 accessions of *T. timopheevii* clustered on distinct branches, and the cladogram showed a similarly topology

Table 1. Chromosome pairing at meiotic metaphase I in pollen mother cells of F_1 hybrids.

Hybrid	Chromosome pairing					
	I	II(total)	II(ring)	II(rod)	III	IV
T. timopheevii ssp. *araraticum* PI427366 × *T. turgidum* ssp. *polonicum* PI210845	8.38(4-15)	9.63(5-12)	3.36(1-5)	6.27(4-8)	0.12 (0-1)	0
T. turgidum ssp. *turanicum* AS2229 × *T. turgidum* ssp. *polonicum* PI366117	0.20(0-2)	13.90(13-14)	1.85(0-6)	12.05(8-14)	0	0
T. turgidum ssp. *durum* Citr14144 × *T. turgidum* ssp. *dicoccoides* AS847	1.84(0-6)	13.08(11-14)	3.94(0-7)	9.14(0-13)	0	0
T. turgidum ssp. *dicoccoides* AS841 × *T.turgidum* L. cv. Ailanmai	1.48(0-6)	13.26(11-14)	6.17(0-12)	7.09(2-10)	0	0
T. turgidum ssp. *dicoccon* PI427274 × *T. turgidum* ssp. *durum* Citr14144	0.88(0-4)	13.56(12-14)	2.66(0-6)	10.90(6-14)	0	0
T. turgidum ssp. *dicoccon* PI427274 × *T. turgidum* ssp. *turgidum* AS2255	0.68(0-4)	13.22(10-14)	1.71(0-5)	11.51(8-14)	0	0.22(0-2)
T. turgidum ssp. *turanicum* PI481582 × *T. turgidum* ssp. *dicoccon* PI427274	0.68(0-4)	13.66(12-14)	2.93(0-7)	10.73(6-14)	0	0
T. turgidum ssp. *dicoccon* PI427274 × *T. turgidum* ssp. *carthlicum* PI387696	0.54(0-2)	13.73(13-14)	1.86(0-5)	11.87(9-14)	0	0
T. turgidum ssp. *turanicum* AS2229 × *T. turgidum* ssp. *durum* Citr14144	0.46(0-4)	13.77(12-14)	2.76(0-4)	11.01(10-14)	0	0
T. turgidum ssp. *turgidum* AS2255 × *T. turgidum* ssp. *polonicum* PI210845	0.20(0-4)	13.44(12-14)	1.52(0-5)	11.92(10-14)	0	0.23(0-1)
T. turgidum ssp. *dicoccon* PI427274 × *T. turgidum* ssp. *dicoccoides* AS841	0.20(0-2)	13.85(13-14)	2.38(0-5)	11.47(9-14)	0	0
T. turgidum ssp. *carthlicum* PI38769 × *T. turgidum* ssp. *durum* Citr14144	0.18(0-2)	13.91(13-14)	1.78(0-5)	12.13(9-14)	0	0
T. turgidum ssp. *turgidum* PI166484 × *T. turgidum* ssp. *carthlicum* PI115816	0.12(0-2)	13.48(12-14)	1.53(0-6)	11.95(9-14)	0	0.23(0-1)
T. turgidum ssp. *carthlicum* PI387696 × *T. turgidum* ssp. *Turgidum* AS2255	0.08(0-2)	13.56(12-14)	1.46(0-6)	12.10(9-14)	0	0.20(0-1)

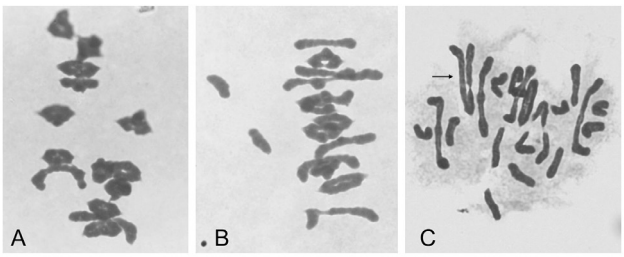

Fig. 6. Meiotic pairing behavior in F_1 hybrids. *A* - *Triticum turgidum* ssp. *dicoccon* (PI427274) × *T. turgidum* ssp. *durum* (CItr14144), 2n = 28 = 12II (Ring) + 2II (Rod); *B* - *T. turgidum* ssp. *durum* (CItr14144 × *T. turgidum* ssp. *dicoccoides* (AS847), 2n = 28 = 2I + 6II (Ring) + 7II (Rod); *C* - *T. timopheevii* ssp. *araraticum* (PI427366) × *T. turgidum* ssp. *polonicum* (PI210845), 2n = 28 = 15I + 1II (Ring) + 4II (Rod) + 1III. The *arrow* indicates the trivalent.

to that of the NJ phylogenetic tree constructed with MEGA. These results indicated that *T. turgidum* was divided into three subclades, which represented potential evolutionary lineages. Importantly, two *T. turgidum* ssp. *dicoccoides* (AS841 and PI470947) and one *T. turgidum* ssp. *dicoccon* (Citr3686) were placed in subclade I. Two *T. turgidum* ssp. *ispahanicum* (PI330548 and PI572904), one *T. turgidum* ssp. *paleocolchicum* (PI349050), and one *T. turgidum*

ssp. *carthlicum* (PI387696) were clustered in subclade II. The remainder accessions of *T. turgidum* were assigned to subclade III, including all *T. turgidum* ssp. *turanicum*, *T. turgidum* ssp. *durum*, one *T. turgidum* ssp. *turgidum* (AS2255), and one T. *turgidum* ssp. *polonicum* (AS304).

The above results did not directly indicate the classification systems of tetraploid wheat. Chromosome pairing in F_1 hybrids of tetraploid wheat was examined to detect genomic affinities (Table 1, Fig. 6). All hybrids derived from *T. turgidum* were of genomic composition AABB, with 2n = 4x = 28. The hybrids of *T. turgidum* ssp. *dicoccoides* showed a high number of univalents in metaphase I cells. The F_1 hybrid of *T. turgidum* ssp. *dicoccoides* × *T. turgidum* L. cv. Ailanmai showed 0 to 6 univalents, with an average of 1.48. The average pairing was 1.84 univalents, 13.08 bivalents in the F_1 hybrid of *T. turgidum* ssp. *durum* × *T. turgidum* ssp. *dicoccoides*. When *T. turgidum* ssp. *turgidum* was a parent, a low frequency of tetravalents was found. These results indicated that hybrids from naked tetraploid wheat showed a high frequency of bivalents and a low frequency of univalents. In the F_1 hybrid of *T. timopheevii* ssp. *araraticum* (PI427366) × *T. turgidum* ssp. *polonicum* (PI210845), meiotic pairing was characterized by a high frequency of univalents ranging from 4 to 15, with an average of 8.38 per cell.

Discussion

Triticum turgidum possesses excellent agronomic traits and is useful as breeding material to transfer target traits to *T. aestivum*. However, the uncertain evolutionary history and classification of tetraploid wheat is not conducive to effective utilization of tetraploid wheat resources. Exploration of the phylogenetic relationships of tetraploid wheat is beneficial to protect the genetic diversity of tetraploid wheat and broaden the genetic basis of *T. aestivum*.

The taxonomy of tetraploid wheat is controversial. *Triticum* has been recognized as a distinct genus in the *Triticeae* since Linnaeus's time (Goncharov *et al*. 2011). As *Aegilops* and *Triticum* are closely related, many taxonomists propose that the two genera should be combined (Yamane and Kawahara 2005). With a clear understanding of the phylogenetic relationship between *Aegilops* and *Triticum*, the B and G genome of *Triticum* were thought to be derived from *Aegilops* (Huang *et al*. 2002). MacKey (1966, 2005) defined *Triticum* to include two intergeneric hybrids of ×*Triticosecale* Wittm, and given that some lines of *Triticum* cross more readily with *Secale* than with *Aegilops*, and that immunochemistry shows *Triticum* and *Secale* to be more similar than *Triticum* and *Aegilops*, thus proposed that *Triticum* and *Secale* should be merged. In the present study, based on network analysis of ITS sequences using *SplitsTree*, all relative species in the *Triticeae* with the exception of *Aegilops* species showed a common topology, whereas tetraploid wheat, *Aegilops* species, and hexaploid wheat were clustered in Clade II. These results showed that *Triticum* and *Secale* were not closely related, thus supporting Bowden's classification (1959) and opposing the treatment of ×*Triticosecale* by MacKey (1966). To further illustrate the relationship between tetraploid wheat and *Aegilops* species, the NJ phylogenetic analysis indicated that tetraploid wheat and *Aegilops* clustered separately on different branches, and *Ae. speltoides* and one accession of *Ae. sharonensis* were more closely related to tetraploid wheat than to other *Aegilops* species. This finding indicated that *Aegilops* plus *Triticum* was not monophyletic, thus rejecting their treatment as a single genus (Bowden 1959).

On the basis of glume morphology, Linnaeus treated tetraploid wheat as *T. polonicum* (Goncharov 2011). With regard to tetraploid wheat, Bowden (1959) recognized one species, *T. turgidum,* three varieties, two variants, and eight cultivars. Given cytogenetic evidence and geographic distribution, Morris and Sears (1967) considered tetraploid wheat to consist of two species. Provan *et al*. (2004) observed that the cytoplasmic types were very similar, supporting the hypothesis for the monophyletic origin of tetraploid wheat. However, Kilian *et al*. (2007) detected a large number of differences between the B and G genomes based on different gene loci, supporting the hypothesis for two independent origins of tetraploid wheat. In the present network analysis and phylogenetic analysis, *T. turgidum* and *T. timopheevii* were placed on different branches, indicating that *T. turgidum* was distantly related to *T. timopheevii.* The FISH signal sites of *T. turgidum* and *T. timopheevii* are very different, and the signal distribution on each chromosome has been mutated. This variation was far greater than the degree of variation among accessions of *T. turgidum*, indicating that *T. turgidum* and *T. timopheevii* show very high genetic differentiation. The F_1 hybrid of *T. timopheevii* ssp. *araraticum* PI427366 × *T. turgidum* ssp. *polonicum* PI210845 showed a high frequency of univalent at metaphase I, which is an indication of genomic incompatibility. These findings indicate that *T. turgidum* and *T. timopheevii* are two distantly related species with different origins.

Numerous classifications of wheats based on different criteria were used by researchers, which has caused ongoing confusion and generic and species concepts remain controversial. Among the two most widely used classifications of tetraploid wheat, MacKey (1966) considered all tetraploid wheat to be subspecies. In contrast, Dorofeev's classification treats all forms of tetraploid wheat as species (Dorofeev *et al*. 1979). The present study of meiotic behavior of F_1 hybrids among tetraploid wheat accessions in metaphase I cells indicated that the frequency of univalents was low (means < 2), and the NJ phylogenetic analysis was consistent with meiotic behavior, which indicated that all forms of *T. turgidum* should not be considered species, thus supporting MacKey's classification system.

Archaeological and genetic evidence suggests that domesticated *T. turgidum* originated from *T. turgidum* ssp. *dicoccoides* in the Fertile Crescent, and that naked tetraploid wheat originated from domesticated *T. turgidum* ssp. *dicoccon* (Zohary *et al*. 2000, Salamini *et al*. 2002, Matsuoka 2011). Tang *et al.* (2017) indicated that

T. turgidum ssp. *dicoccon* and *T. turgidum* ssp. *dicoccoides* grouped a subclade based on the nuclear *DMC1* gene, and concluded that at least two intermediary subspecies were involved in the evolution of *T. turgidum.* The F_1 hybrids of *T. turgidum* ssp. *dicoccoides* showed a higher frequency of univalents than other hybrids. In the cluster analysis of FISH results, two *T. turgidum* ssp. *dicoccoides* (TDI AS841 and TDI PI470947) and one *T. turgidum* ssp. *dicoccon* (TDC Citr3686) were clustered in subclade I. These results show the special status of *T. turgidum* ssp. *dicoccoides* in *T. turgidum. Triticum turgidum* ssp. *dicoccoides* contains two populations, one from the west, including Jordan, Israel, Lebanon, and Syria, and the other from the Middle East, including Iran, Iraq, and Turkey. Many studies have shown that *T. turgidum* ssp. *dicoccoides* in the Middle East is the ancestral species of domesticated tetraploid wheat (Özkan *et al*. 2005, 2011, Matsuoka *et al*. 2011, Oliveira *et al*. 2012). In the present study, accessions of *T. turgidum* ssp. *dicoccoides* from the western population clustered with domesticated tetraploid wheat, which is inconsistent with the results of Özkan *et al*. (2011) and Oliveira *et al*. (2012). With regard to meiotic behavior of F_1 hybrids, and the results of NJ phylogenetic analysis and network analysis, the forms of *T. turgidum* were not distinguished except for *T. turgidum* ssp. *dicoccoides*. Therefore, we advocate continued recognition of *T. turgidum* ssp. *dicoccoides* as a subspecies and the treatment of other forms of *T. turgidum* as varieties.

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