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RESEARCH ARTICLE

Novel and ancient HMW glutenin genes from *Aegilops tauschii* and their phylogenetic positions

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Abstract A pair of novel high-molecular-weight glutenin subunits (HMW-GS) 1Dx3.1^{t} and 1Dy11*^{t} were revealed and characterized from *Aegilops tauschii* Coss. subspecies *tauschii* accession AS60. SDS-PAGE band of 1Dx3.1^{t} was between those of 1Dx2 and 1Dx3, while 1Dy11*^{t} was between 1Dy11 and 1Dy12. The lengths of *1Dx3.1^{t}* and *1Dy11*^{t}* were 2,514 bp and 1,968 bp, encoding 836 and 654 amino acid residues, respectively. Their authenticity was confirmed by successful expression of the coding regions in *Escherichia coli*. Network analysis indicated that *1Dx3.1^{t}* together with other five rare alleles only detected in Asia common wheat populations represented the ancestral sequences in *Glu-D1* locus. Neighbor-joining tree analysis of previously cloned x-type and y-type alleles in the *Glu-D1* locus supported the hypothesis that more than one *Ae. tauschii* genotypes were involved in the origin of hexaploid wheat and that different *Ae. tauschii* accessions contributed the D genome to common wheat and *Ae. cylindrical* Host, respectively. An *Ae. tauschii* accession with *1Dx3.1^{t}* or a closely related allele probably have involved in the origin of common wheat. Since accession AS60 used in this study belonged to typical ssp. *tauschii*, present results suggested the possibility that ssp. *tauschii* was involved in the evolution of common wheat.

Keywords *Aegilops tauschii* · Bacterial expression · HMW-GS · Phylogenetic analysis

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Introduction

High-molecular-weight glutenin subunits (HMW-GSs) play an important role in determining dough viscoelastic properties and bread-making quality of common wheat (*Triticum aestivum* L.) (Payne 1987; Shewry et al. 1992). HMW-GSs are encoded by complex *Glu-1* loci, which are located on the long arms of homoeologous group 1 chromosomes. Each *Glu-1* locus consists of two paralogous alleles of duplication origin that encode the x and the y types of HMW-GSs, respectively. The *Glu-1* loci on chromosomes 1A, 1B and 1D are called *Glu-1A*, *Glu-1B* and *Glu-1D*, respectively. Therefore, common wheat has six HMW-GS alleles, namely *Glu-1Ax*, *Glu-1Ay*, *Glu-1Bx*, *Glu-1By*, *Glu-1Dx* and *Glu-1Dy* (Payne 1987; Shewry et al. 1992).

Aegilops tauschii Coss. is the D-genome progenitor of common wheat. Some HMW-GS alleles from *Ae. tauschii* have been integrated into hexaploid wheat background and made a positive influence on the bread-making properties (Peña et al. 1995; Hsam et al. 2001; Wieser et al. 2003). *Ae. tauschii* has a much higher HMW-GS diversity than common wheat on *Glu-D1* locus (Payne and Lawrence 1983; Lagudah and Halloran 1988, 1989; Lagudah and Appels 1993; William et al. 1993; Gianibelli et al. 2001; Yan et al. 2003; Rehman et al. 2008). To date, 12 new x-type alleles (*1Dx1.1*^{t}, *1Dx1.5*^{t}, *D*^{t}*x1.5*, *1Dx1.6*^{t}, *1Dx2*^{t}, *1Dx2.1*^{t}, *1Dx3*^{t}, *1Dx4*^{t}, *Dx5*^{t}, *1Dx5**^{t}, *1Dx5.1**^{t}, and *1Dx5.2*^{t}) (Lu and Lu 2005; Wan et al. 2005; Zhang et al. 2008; Yan et al. 2008; An et al. 2009; Fang et al. 2009) and 15 new y-type alleles (*Dy8.1*^{t}, *1Dy9.5*, *D* ^{t}*y10*, *1Dy10.1*^{t}, *1Dy10.4*^{t}, *1Dy10.5*^{t}, *1Dy10.5**^{t}, *Dy12*^{t}, *1Dy12*^{t}, *1Dy12.1*^{t}, *1Dy12.1**^{t}, *1Dy12.2*^{t}, *Dy12.4* ^{t}, *1Dy12.5* ^{t}, and *1Dy13*^{t}) (Mackie et al. 1996; Yan et al. 2002, 2004, 2008; Hassani et al. 2005; Lu and Lu 2005; Zhang et al. 2006, 2009; Su et al. 2009; Chang et al. 2010) have been sequenced in *Ae. tauschii*.

This communication reports discovery and characterization of novel x- and y-type HMW-GS alleles from *Ae. tauschii* subspecies *tauschii* accession AS60 and discusses their phylogenetic relationships with previously identified alleles on the *Glu-D1* loci of *T. aestivum* and *Ae. tauschii*.

Materials and methods

Plant materials and SDS-PAGE

Plant materials used in this study included *Ae. tauschii* (2n = 2x = 14, DD) subspecies *tauschii* accession AS60 from Iran and common wheat (2n = 6x = 42, AABBDD) lines Chinese Spring (null, Bx7 + By8, Dx2 + Dy12), Longfumai 1 (1Ax2*, Bx7 + By8, Dx5 + Dy10), Jinan 17 (1Ax1, Bx7 + By8, Dx3 + Dy12), and Shannongfu 63 (?, Bx7 + By9, Dx4 + Dy11). The common wheat cultivars were used as standards in the comparisons of electrophoresis mobility of HMW-GSs. Seed protein extraction and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were done as described by Wan et al. (2005).

DNA extraction and PCR amplification

Genomic DNA was extracted from young leaves of *Ae. tauschii* accession AS60 as described by Yan et al. (2002). A pair of universal primers (P1: 5′-ATGGC TAAGCGGTTAGTCCTCTTTG-3′, P2: 5′-CTAT CACTGGCTGGCCGACAATGCG-3′) was designed and used for amplifying the complete coding region of the *Glu-D1* alleles following the method by Yuan et al. (2009), which was modified from Sugiyama et al. (1985) and Anderson et al. (1989).

Cloning, sequencing and comparative analyses of *Glu-D1* genes

PCR products were cut from agrose gel and purified using E.Z.N.A.®Gel Extraction Kit (OMIGA). The DNA fragments were then ligated into pMD18-T plasmid vector (TaKaRa, Dalian, China). The ligated products were transformed into *Escherichia coli* strain DH10B and then sequenced from the plasmids. Full-length sequence of each allele was obtained by sequencing a set of overlapping sub-clones with nested deletion method (Yan et al. 2002). Multiple sequences were aligned using ClustalX with default options (Thompson et al. 1999) and the alignments were manually refined in an effort to maximize the positional homology.

Expression of cloned *Glu-D1* alleles in *E. coli*

The cloned *Glu-D1* alleles were amplified to remove the signal peptides using the primers: 5′-ACC CATATGGAAGGTGAGGCCTCTGAGC-3′ and 5′-T TCCTCGAGCTATCACTGGCTGGCCGAC-3′ for *1Dx3.1*^{t}, and 5′-ACCCATATGGAAGGTGAGGCC TCTAGGC-3′ and 5′-TTCCTCGAGCTATCACTGG CTGGCCGAC-3′ for 1Dy11*^{t} (*Nde*I or *Xho*I restriction site is underlined). The modified PCR products were cloned into the expression vector pET-30a (Novagen) and transformed into *E. coli* strain *BL21 (DE3) pLsS*. Expression of HMW-GSs in *E. coli* was induced with 1 mM isopropyl *β*-Δ-thiogalactopyranoside (IPTG) for 5 h using the cells without IPTG as reference. Samples of the expressed HMW-GSs for *1Dx3.1*^{t} were extracted from 1 ml of the induced or the uninduced bacterial cultures and used for SDS–PAGE analysis according to Wan et al. (2005). The

selectively precipitated protein samples for 1Dy11*^{t} were prepared for SDS–PAGE analysis as described by Hu et al. (2010).

Phylogenetic analysis

Median-joining (MJ) network was constructed as described by Fan et al. (2009) for this study using the nucleotide sequences of signal peptide, N-terminal domain, and C-terminal domain. Neighbor-joining tree was constructed by the software MEGA5. The bootstrap values in phylogenetic tree were estimated based on 1,000 replications. Tajima's π (Tajima 1989) and Watterson's θ (Watterson 1975) were conducted to estimate nucleotide diversity. Tajima's π quantifies the mean percentage of nucleotide differences among all pairwise comparisons for a set of sequences, while Watterson's θ is simply an index of the number of segregating (polymorphic) sites. These parameters were calculated with DnaSP 4.10.9 (Rozas et al. 2005) and ProSeq 2.0 (Filatov 2002).

Results and discussion

SDS-PAGE analysis

SDS-PAGE analysis showed that *Ae. tauschii* accession AS60 possessed a pair of novel HMW-GSs (Fig. 1a). The band of the new x-type subunit was situated between those of 1Dx2 and 1Dx3. This new x-type HMW-GS thus was designated as 1Dx3.1^t. The new y-type subunit was situated between 1Dy11 and 1Dy12, and, therefore, was designated as 1Dy11*^{t}.

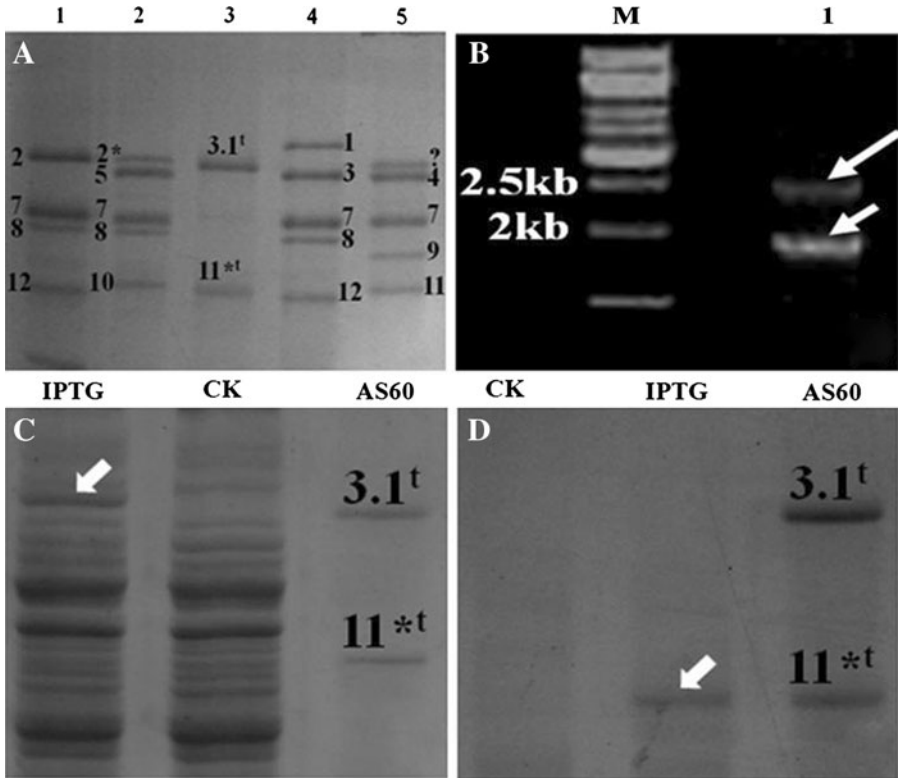

Fig. 1 Characterization of HMW-GS alleles in *Ae. tauschii* AS60.**a** SDS-PAGE detection of HMW-GSs in Chinese Spring (*lane 1*), Longfumai 1 (*lane 2*), *Ae. tauschii* accession AS60 (*lane 3*), Jinan 17 (*lane 4*), and Shannongfu 63 (*lane 5*). **b** PCR amplification of alleles *1Dx3.1^t* (*long arrowhead*) and *1Dy11*^{t} (*short arrowhead*) from AS60. **c** Expression of the cloned *1Dx3.1^t* in *E. coli*. The expression of mature protein was detected in the IPTG-induced bacterial cells (*arrowhead*), which showed a band identical to 1Dx3.1^t extracted from seed from AS60. In contrast, this mature protein was not observed in the control bacterial culture (CK) that was not induced by IPTG. **d** Expression of the cloned *1Dy11*^{t} in *E. coli*. The bacterially expressed mature protein (*arrowhead*) showed a band identical to 1Dy11*^{t} from AS60

Molecular characterization of novel HMW-GS genes in AS60

PCR amplification of the coding region of the two new HMW-GS genes from *Ae. tauschii* AS60 resulted in products of about 2,500 bp (Fig. 1b, long arrowhead) for x-type and 2,000 bp for y-type subunit, respectively (Fig. 1b, short arrowhead). Sequences of the two DNA fragments for *1Dx3.1*^{t} and *1Dx11**^{t} were 2,514 and 1,968 bp in length, respectively. Their putative full encoding sequences were comprised of 836 and 654 amino acids. The authenticity of the cloned x-type (Fig. 1c) and y-type (Fig. 1d) genes was confirmed by successful expression of the encoding regions in *E. coli.* The sequences of the two novel *Glu-D1* alleles were deposited in Genbank under accession number HM124446 and HM124445.

Comparison of genes in AS60 with others in *Glu-D1*

Sequence analysis indicated that *1Dx3.1*^{t} had similar primary structure to other known D-genome encoded x-type alleles. It contained four domains: a signal peptide (21 amino acids), a conserved N-terminal domain (89 amino acids), a C-terminal domain (42 amino acids), and a repetitive domain made up by repetitive motifs of tripeptide (GQQ), hexpeptide (PGQGQQ), and penapeptide (GYYPTSPQQ). Compared to previously reported *Glu-D1* alleles *D*^{t}*x1.5* (AY594355), *1Dx1.5*^{t} (EF546438), *1Dx2*^{t} (AF480485), *1Dx2.1* (AY517724), *1Dx2.1*^{t} (AF480486), *1Dx4*^{t} (DQ307383), *1Dx5*^{t} (HM050419), and *1Dx5.2*^{t} (DQ307384), *1Dx3.1*^{t} had three unique SNPs at the 785, 1,712, and 1,842 bp positions in the repetitive domain. Nucleotide G existed in *1Dx3.1*^{t}, while A existed in others at the three positions. The former two SNPs resulted in amino acid substitution (glutamine → arginine).

*1Dy11**^{t} also showed similar primary structure to other y-type alleles at the *Glu-D1* locus. Compared to *1Dy* (AJ306974), *Dy8.1*^{t} (FJ3998041), *1Dy9.5* (FJ008134), *1Dy10.4*^{t} (FJ499504), *1Dy10.5**^{t} (FJ499502), and *1Dy10.5*^{t} (FJ499503), five unique SNPs were found in *1Dy11**^{t} at positions of 121 bp (G → A, glutamate → lysine), 282 bp (G → A), 857 bp (A → G, glutamine → arginine), 1,431 bp (A → C, glutamine → histidine), and 1,919 bp (C → T, threonine → isoleucine).

Phylogenetic relationships of HMW-GS genes based on neighbor-joining trees

A neighbor-joining tree was constructed with complete amino acid sequences of 12 x-type and 11 y-type genes in loci *Glu-A1*, *Glu-B1*, and *Glu-D1*. In the tree, x- and y-type alleles were apparently divided into two lineages (Fig. 2a). Obviously, x- and y-type alleles were diverged from an ancestral sequence prior to the separation of the wheat genomes A, B, and D (Shewry et al. 1989). *1Dx3.1*^{t} and *1Dy11**^{t} in AS60 were clustered together with the previously reported x-type and y-type alleles in locus *Glu-D1*, respectively.

In order to further investigate evolution of the *Glu-D1* alleles, we constructed a neighbor-joining tree using complete coding sequences of 48 alleles in locus *Glu-D1*, including 24 x-type and 24 y-type alleles (Fig. 2b). Of them, 13 x-type and 16 y-type alleles were from *Ae. tauschii*, one y-type from species *Ae. cylindrica*, and the others from common wheat. Again, the x-type and y-type alleles were apparently divided into two clades. The x-type alleles were further separated into two clusters, which comprised of *1Dx1.1*^{t} at the bottom and the other 23 at the top. *1Dx1.1*^{t} is the largest HMW-GS allele reported so far in this locus of *Ae. tauschii* (Fang et al. 2009).

The y-type alleles formed four clades. The y-type alleles of the common wheat cultivars and *Ae. tauschii* fell into the same two clades (III and IV). *1Dy10* and *1Dy12* widely exist in current common wheat lines. *1Dy10* and five *Dy* genes from *Ae. tauschii* (*1Dy12*^{t}, *Dy12*^{t}, *1Dy12.1*^{t}, *1Dy12.2*^{t}, *and 1Dy12.1**^{t}) formed clade III. *1Dy12* was clustered in clade IV with five common wheat alleles (*1Dy12.3, 1Dy11, 1Dy12.2**, *1Dy12**, and *1Dy10.1*) and two *Ae. tauschii* alleles (*D*^{t}*y10* and *1Dy10.1*^{t}) (Fig. 2b). A follow-up MJ network analysis also agreed with this result (Fig. 3b). It seems that that *1Dy10* and *1Dy12* were derived from different *Ae. tauschii* accessions, thus supporting the hypothesis that more than one *Ae. tauschii* genotypes have been involved in the polyploidization process during the speciation of common wheat (Dvorák et al. 1998; Talbert et al. 1998; Lelley et al. 2000; Caldwell et al. 2004; Giles and Brown 2006; Zhang et al. 2008; Fang et al. 2009).

The y-type gene *1Dy11**^{t} in AS60 was clustered in clade II together with *1Dy10.4*^{t}, *1Dy10.5*^{t}, *1Dy10.5**^{t}, *1Dy9.5*, *Dy12.4*^{t}, and *Dy8.1*^{t} from *Ae. tauschii*, and *1Dy* (AJ306974) from *Ae. cylindrica* (Fig. 2b). None

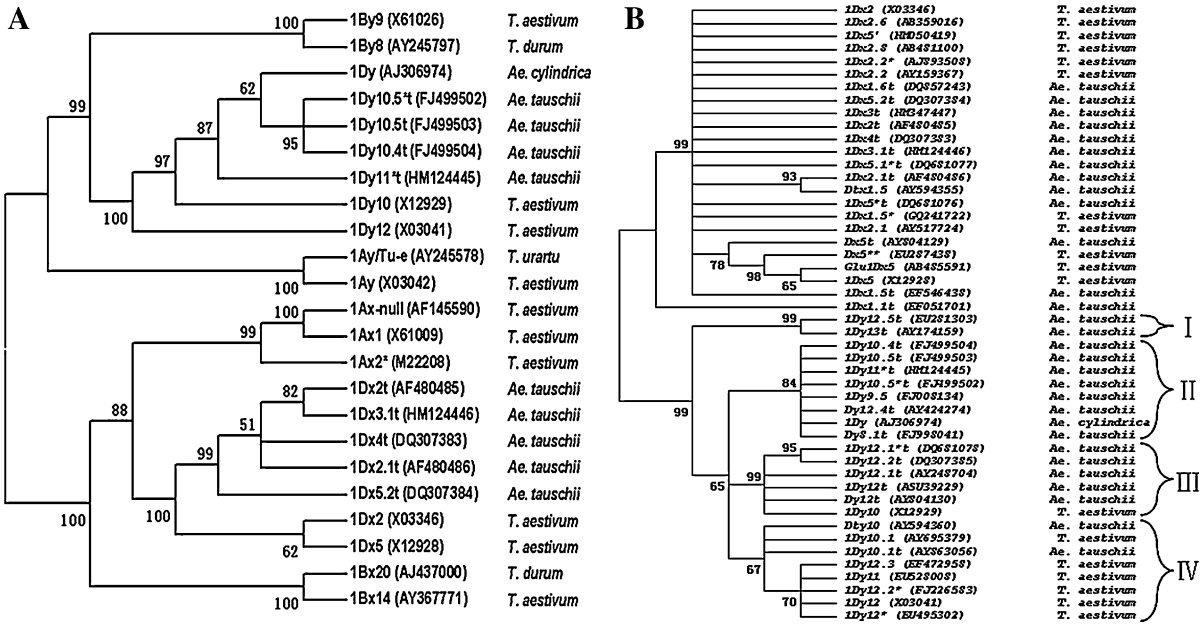

Fig. 2 **a** The neighbor-joining tree based on complete amino acid sequences of 12 x-type and 11 y-type alleles in loci *Glu-A1*, *Glu-B1*, and *Glu-D1*. **b** The neighbor-joining tree based on complete coding sequences of 24 x-type and 24 y-type alleles in locus *Glu-D1*. The bootstrap values are indicated near nodes. GenBank accession numbers are in *brackets* after the alleles. Genbank accession number HM124446 and HM124445 for *1Dx3.1*^{t} and *1Dx11**^{t} from this study, others from previously reported references (Mackie et al. 1996; Yan et al. 2002, 2004, 2008; Hassani et al. 2005; Lu and Lu 2005; Wan et al. 2005; Zhang et al. 2006, 2008, 2009; An et al. 2009; Fang et al. 2009; Su et al. 2009; Chang et al. 2010)

of the common wheat alleles was included in this cluster. This result supported the result by Caldwell et al. (2004) that different *Ae. tauschii* accessions contributed the D genome to common wheat and *Ae. cylindrica*.

Gene genealogies based on network analysis

For genealogy analysis, MJ networks were constructed with nucleotide sequences of signal peptide, N-terminal domain, and C-terminal domain of 24 *1Dx* (Fig. 3a) and 24 *1Dy* alleles (Fig. 3b). MJ network is an effective method to study gene genealogies (Cassens et al. 2005; Kilian et al. 2007). The network placed the six alleles *1Dx3.1*^{t} (this study), *1Dx1.5** (Guo et al. 2010), *1Dx2.2** (Wan et al. 2005), *1Dx5′* (Feng et al. 2011), *1Dx2.6* (Cong et al. 2007), and *1Dx2.8* (Terasawa et al. 2009) at a principal node (Fig. 3a), while the other alleles radiated in a star-like phylogeny from the principal node. The six alleles might represent the ancestral sequences. It is interesting that *1Dx3.1*^{t} was among the six alleles and the other five all were rare and only detected in Asia common wheat lines. The sequence of *1Dx2.8*, detected in an Afghan landrace, is the shortest of the known Dx-type alleles (Terasawa et al. 2009), while *1Dx2.6*, observed in some Xinjiang landraces in China, is the longest (Cong et al. 2007). Chinese common wheat line W958 from Sichuan has *1Dx5′* (Feng et al. 2011) and Jiuquanjinbaoyin from Gansu contains *1Dx1.5** (Guo et al. 2010). Wheat line MG315, which has *1Dx2.2**, was also collected from China (Personal communication with professor Renato D'Ovidio of Universita' degli Studi della Tuscia).

These six putative ancestral alleles were then compared with each other for sequence polymorphism, and found to be identical in the signal peptide, N-terminal domain, and C-terminal domain. The only differences appeared in the repetitive domain, including SNPs and size variations due to insertions/deletions (Fig. 4). *1Dx2.6*, *1Dx2.2**, *1Dx5′*, and *1Dx1.5** contained a same indel with 27 bp at position of 1,847 bp. *1Dx2.8* contained two indels with 261 and 585 bp. Both *1Dx1.5** and *1Dx3.1*^{t} contained two indels with 18 bp and 576 bp. The sequence similarity suggested that an *Ae. tauschii* accession (such as

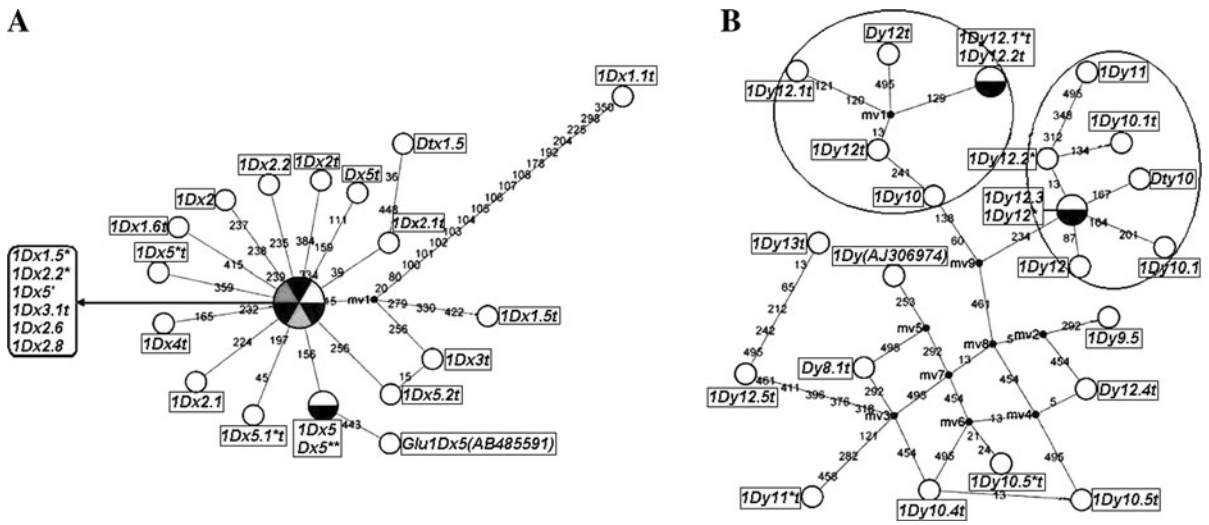

Fig. 3 Median-joining networks based on nucleic acid sequences of signal peptide, N-terminal domain, and C-terminal domain of 24 1Dx (**a**) and 24 1Dy alleles (**b**), respectively. *mv* denotes a hypothetical haplogroup not found in this study. Alleles in network are represented by circles. *Numbers* above *line* are mutation sites between the two linked alleles

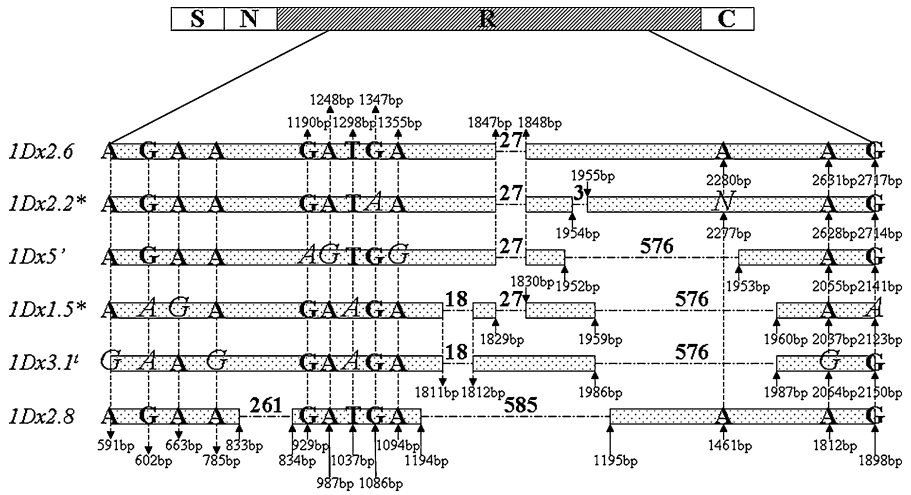

Fig. 4 Comparison of nucleic sequences among six ancestral x-type alleles in locus *Glu-D1* that share identical signal (S), N-terminal domain (N), and C-terminal domain (C). Variations such as SNPs and indels occurred in the repetitive domain (R). Different *letters* represent SNPs. *Numbers on dash dot lines* show the size of deletions

AS60) that contains allele *1Dx3.1*^{t} or a closely related allele might have also involved in the origin of common wheat that has allele *1Dx1.5**. The neighbor-joining tree based on complete amino acid sequences also supported this hypothesis (Fig. 5).

Aegilops tauschii was classified into two subspecies, i.e. ssp. *tauschii* with elongated cylindrical spikes and *strangulata* (Eig) Tzvel. with markedly moniliform spikes (Hammer 1980). However, morphologically intermediate forms between the two typical subspecies had been also observed (Matsuoka et al. 2009 and cited references). Previous results indicated that ssp. *strangulata* or intermediate form was the D-genome donor of common wheat (Dvorák et al. 1998; Huang et al. 2011). However, accession AS60 used in this study belonged to typical ssp.

Fig. 5 A neighbor-joining tree constructed from complete amino acid sequences of six ancestral x-type alleles in locus *Glu-D1*. GenBank accession *numbers* are in *brackets* after the alleles

Table 1 Estimates of nucleotide diversity at *Glu-D1* locus in *T. aestivum* and *Ae. tauschii*

	n	*s*	π	θ_w
Triticum aestivum				
Dx	456	4	0.0022	0.0030
Dy	501	10	0.0061	0.0082
Aegilops tauschii				
Dx	456	26	0.0100	0.0187
Dy	501	27	0.0131	0.0159

The *n* is the number of the sites (excluding sites with gaps), *s* is the number of segregating sites, π is the average of diversity between any two genes in a set of sequences, and θ_w is the diversity based on the number of segregating sites

tauschii (Huang et al. 2011). Present results suggested the possibility that ssp. *tauschii* was also involved in the evolution of common wheat.

Unlike 1Dx alleles, our analysis did not reveal any apparent principal node among the 24 1Dy alleles (Fig. 3b). This result suggested that Dx-type and Dy-type genes have evolved independently and Dy genes had faster evolutionary rates than Dx genes. This hypothesis was supported by estimated nucleotide polymorphism (Table 1). Nucleotide polymorphic parameters among *Glu-1Dy* alleles were higher than those among *Glu-1Dx* alleles except for θ_w in *Ae. tauschii*. The higher θ_w of *Glu-1Dx* than *Glu-1Dy* may be from the large differentiation contributed by allele *1Dx1.1*^{t} (Fig. 2b and 3a; Fang et al. 2009). Generally, nucleotide polymorphism in *Ae. tauschii* is higher than *T. aestivum*.

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