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ORIGINAL PAPER

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GluDy allele variations in *Aegilops tauschii* and *Triticum aestivum*: implications for the origins of hexaploid wheats

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Abstract To investigate the evolution and geographical origins of hexaploid wheat, we examined a 284 bp sequence from the promoter region of the *GluDy* locus, coding for the *y* subunit of high-molecular-weight glutenin. Fourteen different alleles were found in 100 accessions of *Aegilops tauschii* and 169 of *Triticum aestivum*. Two alleles were present in both species; the other 7 alleles from *Ae. tauschii* and 5 from *T. aestivum* were unique to their respective species. The two shared alleles differed at only one nucleotide position within the region sequenced, but their apparent association with the common haplotypes *GluD1a* and *GluD1d*, which have substantial differences within their *GluDy* coding regions, makes it unlikely that the alleles evolved independently in *Ae. tauschii* and *T. aestivum*. The results therefore support previous studies which suggest that there were at least two *Ae. tauschii* sources that contributed germplasm to the D genome of *T. aestivum*. The number of alleles present in *T. aestivum*, and the nucleotide diversity of these alleles, indicates that this region of the D genome has undergone relatively rapid change since polyploidisation. *Ae. tauschii* from Syria and Turkey had relatively high nucleotide diversity and possessed all the major *GluDy* alleles, indicating that these populations are probably ancient and not the result of adventive spread. The presence in the Turkish population of both of the shared alleles suggests that hexaploid wheat is likely to have originated in southeast Turkey or northern Syria, within the Fertile Crescent and near to the farming villages at which archaeological remains of hexaploid wheats are first found. A second, more recent, hexaploidisation probably occurred in Iran.

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Introduction

Hexaploid wheat (*Triticum aestivum* L., genomic constitution AABBDD) arose by amphiploidisation between tetraploid wheat [*T. turgidum* L. ssp. *dicoccum* (Schrank) Thell., AABB] and diploid goat grass (*Aegilops tauschii* Coss., DD) (Kihara 1944; McFadden and Sears 1946a, b). Hexaploid wheats are not normally found in the wild and are thought to have evolved from cultivated tetraploid wheat (Zohary and Hopf 2000). Increasing evidence suggests that this event occurred at least twice and that hexaploid wheats are polyphyletic. This was first suggested by two-dimensional electrophoresis of high-molecular-weight (HMW) glutenin proteins (Lagudah and Halloran 1988), restriction fragment length polymorphism (RFLP) analysis (Dvorak et al. 1998a), polymerase chain reaction (PCR) of sequence tagged sites (Talbert et al. 1998), and microsatellite analysis (Lelley et al. 2000). Further evidence has recently been provided by examination of single nucleotide polymorphisms (SNPs) at the *Xwye838* and *Gss* loci, coding for ADP-glucose pyrophosphorylase and granule-bound starch synthase, respectively, both of which exist as two distinct variants in hexaploid wheat (Caldwell et al. 2004).

Although the evolutionary origins of hexaploid wheat are becoming better understood, the geographical origins of the species are much less certain. The available evidence is based mainly on biogeography and genetic analysis. Biogeography suggests that the ranges of *Ae. tauschii* and *T. turgidum* did not overlap until domesticated forms of the latter began to spread with the expansion of agriculture (Zohary and Hopf 2000). This is because wild *T. turgidum* is confined to the Fertile Crescent, being found mainly in Israel, Jordan, Syria, Lebanon, south-eastern Turkey, north Iraq and west Iran, whereas the primary distribution of *Ae. tauschii* is in central Asia—in north Iran, Transcaucasia, Transcaspia and Afghanistan. Today, there are also peripheral populations of *Ae. tauschii* within the Fertile Crescent,

but this is said to result from adventive spread associated with farming (Zohary and Hopf 2000), *Ae. tauschii* being a highly successful coloniser of secondary habitats including wheat fields and road edges. Adventive spread with the expansion of farming was most likely responsible for the eastward movement of *Ae. tauschii* to the vicinity of the Yili River and Henan Province in China, but a *westward* movement is less easy to reconcile with cultivation, and it has been suggested that the westernmost populations are in fact part of the natural continuous distribution zone of *Ae. tauschii* (van Slageran 1994).

Any role for these peripheral populations in the origins of hexaploid wheat have been largely discounted because most of the available genetic information supports the hypothesis that hexaploidisation occurred after cultivated *T. turgidum* had spread into the primary distribution zone of *Ae. tauschii*. Comparisons of protein markers in *Ae. tauschii* and wheat by Jaaska (1980, 1981) and Lagudah et al. (1991) suggested that *Ae. tauschii* ssp. *strangulata* was the most likely donor of the wheat D genome. However, the material used by Jaaska was not representative of the *Ae. tauschii* genepool and the results of Lagudah et al. suggested that 10% of hexaploid wheat did not share the combination of markers found in ssp. *strangulata*. Studies by Tsunewaki et al. (1966, 1991) and Nishikawa et al. (1980) also suggested that ssp. *strangulata* could have been the D genome donor, but again the work was not conclusive. All these studies, and that of Nakai (1979), have suggested that the birthplace of hexaploid wheat was most likely to lie within the region comprising Transcaucasia, northern Iran and the south Caspian coast. This region coincides with the known distribution zone for ssp. *strangulata*, supporting the assumption that this subspecies is the D genome donor.

Contrary views have been put forward by Lelley et al. (2000), who compared microsatellite loci of hexaploid wheat with those found in *Ae. tauschii* and concluded that ssp. *tauschii* from Georgia is the most likely donor of the D genome, and by Morris et al. (2003), who also found that ssp. *tauschii* was more similar to the D genome of *T. aestivum* when puroindoline genes (controlling 'hardness' of grain) were compared. Despite these inconsistencies, ssp. *strangulata* is still cited as the most likely donor of the D genome. At first it was thought that this conclusion provided a specific indication of the geographical origin of hexaploid wheat, ssp. *strangulata* being confined to Transcaucasia and the Caspian region, but Dvorak et al. (1998a) discovered that the '*strangulata*' genepool also includes plants which, on morphological grounds, belong to ssp. *tauschii*. The latter subspecies is more widespread than ssp. *strangulata*, though Dvorak et al. (1998a) concluded from RFLP studies that the D genome of hexaploid wheat is most closely related to the '*strangulata*' genepool present in Transcaucasia (Armenia in particular) and southwest Caspian Iran.

The genetic evidence therefore lends support to the view that hexaploid wheat evolved in Transcaucasia and/or the South Caspian region after the spread of cultivated tetraploid wheat reached those areas, between 6000 and 5000 bc (van Zeist 1976; Zohary and Hopf 2000). It must be noted, however, that the centre of diversity for *Ae. tauschii* is also Transcaucasia and the South Caspian region (van Slageran 1994), making the presence of ancestral alleles and traits in these populations highly probable, a situation which would substantially weaken much of the genetic information. It has also been pointed out that the archaeological evidence is inconsistent with hexaploid origins in Transcaucasia and the South Caspia (Nesbitt and Samuel 1996). The earliest records of hexaploid wheat date to 6800–6400 bc, from Cafer Höyük, Can Hasan III and Çatalhöyük in Turkey and Abu Hureyra in Syria (Hillman 1978; Moore et al. 2000; de Moulins 1993, 2000; Fairbairn et al. 2002). While accepting that the archaeobotanical record is incomplete, and that overlaps in morphological traits may lead to preserved remains of tetraploid and hexaploid wheats occasionally being mistyped, this contrary view from archaeology cannot be easily dismissed.

The questions regarding the origins of hexaploid wheats could be resolved by phylogeographical analysis of *Ae. tauschii*, including identification of the geographical regions within which genotypes present in hexaploid wheats are found. Most previous attempts to do this have used protein expression or RFLP data, but it is now recognised that such 'whole-genome' approaches might not yield accurate information because of the confusing effects of the genome restructuring that occurs after polyploidisation (reviewed by Liu and Wendel 2002). To minimise this problem, it will be necessary to base phylogeographical studies on single, informative loci which display shared polymorphism in *T. aestivum* and *Ae. tauschii*, this type of analysis being further justified by the relatively low likelihood of introgression involving D genome loci compared with those on the A and B genomes (Dvorak et al. 1998a). The best documented shared polymorphism is at the HMW glutenin locus *Glu1*, coding for a seed storage protein. This locus encodes two subunits from two tightly linked paralogous genes denoted *x* and *y*, each with its own promoter. Hexaploid wheat has six known *GluD1* haplotypes (Payne and Lawrence 1983), and two of these, *GluD1a* (peptide subunits 2 and 12) and *GluD1d* (subunits 5 and 10) have also been found in *Ae. tauschii* (Lagudah and Halloran 1988). The open reading frames of the *Glu1* loci contain highly repetitive sequence motifs that make phylogenetic analysis difficult, but in previous work we showed that the promoter regions contain sufficient phylogenetic information to enable alleles to be distinguished, and that comparisons between promoter sequences can provide information on the evolution of the *Glu1* loci (Allaby et al. 1999). We therefore examined SNPs in a 284 bp segment of the *GluDy* promoter in *Ae. tauschii* accessions and *T. aestivum* landraces and cultivars in order to investigate the evolution and geographical origins of hexaploid wheat.

Materials and methods

Plant material consisted of 100 accessions of *Ae. tauschii* and 169 of *T. aestivum*, obtained from: the Institute of Plant Science Research (IPSR) Collection of Wheat and Related Species, John Innes Centre, Norwich, UK; the National Small Grains Research Facility (NSGRF), Idaho, USA; the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany; the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria; and Dr. J. Raupp, Kansas State University, USA. The *Ae. tauschii* accessions were from across the geographical range of the species. Only limited information was available regarding subspecies or the specific details regarding the collection sites. The *T. aestivum* accessions comprised 116 accessions of *T. aestivum* ssp. *aestivum*, 19 accessions of ssp. *spelta* (L.) Thell. (8 Asian, 11 European), 17 ssp. *compactum* (Host) MacKey, 9 ssp. *macha* (Dekr. et Men.) MacKey, 7 ssp. *sphaerococcum* (Perc.) MacKey and 1 ssp. *vavilovi* (Tuman.) MacKey. The *T. aestivum* ssp. *aestivum* accessions included 9 cultivars: Chinese Spring, Hobbit, Champlein, Hope, Flinor, Danchi, Norin 10, Neepawa and Sicco, the first six of these being variety standards for the different glutenin haplotypes (Payne and Lawrence 1983).

A nested PCR system was designed to amplify a partial promoter sequence from the *GluDy* locus (Fig. 1a). The initial PCR, with primers P1 and P2, was not genome specific and yielded a mixed PCR product derived from all six copies of the HMW glutenin genes of the A, B and D genomes. The nested PCR, with primers P3 and P4, amplified only the *GluDy* locus. Primers P3 and P4 were designed by alignment of published allele sequences to identify mismatched regions (Fig. 1b) and tested for *GluDy* specificity with AA and DD diploid accessions, AABB tetraploids and AABBDD hexaploids. Primer sequences were: P1, 5′-TGCCAAACCCCAAGAAG-3′; P2, 5′-ACGAGGGC-GATGACTAC-3′; P3, 5′-AAACAATACCCA-GAAGCCA-3′; P4, 5′-TGCCGCAAAGAGGACCAG-3′. It is possible to achieve specific amplification of *GluDy* with a single PCR using P3 and P4, but in our hands the nested system was more efficient. Rare or unexpected sequence features, which might arise during the multiple cycles of a nested PCR, were checked by PCR with a second aliquot of the DNA extract.

Nuclear DNA was extracted from single seeds using the High Pure PCR Product Purification kit (Roche) and eluted in 150 µl of elution buffer. 'Hot start' PCR (Erlich et al. 1991) was performed in 100 µl reaction mixes containing 9 µl of extract and 200 ng each of primers

(a)

```
            P1 >                                                                1              P3 >
CTGCCAAACCCCAAGAAGCATAACCACTTCTCTTAGATAAAAATAGCAGATCGATATACAAACGGTCTACAACTTCTGCAAACAATACCCAGAAGCCAGAATTAGG
                  50                                                  100
ATTGAACCGATTACGTGGCTTTAGCAGACCGTCCAAAAATCTGTTTTTGCAAAGCTCCAATTGCTCCTTGCTTATCCAGCTTCTTTTTGTGTTGGCAAATTGTTCTTT
              150                                                 200
TCCAACCGACTTTATTCTTTTCACATTTCTTCTTAGGCTGAACTAACCTCGTCGTCGACACAACCATTGTCCTGAACCTTCACCACGTCCCTATAAAAGCCCAACC
         250                                             300                                  < P4
AATCTCCACAATTTCATCATCACCCACAACACCGAGCACCACAAAATAGAGATCAATTCACTGACAGTCCACCGAGATGGCTAAGCGGCTGGTCCTCTTTGCGGCA
< P2
GTAGTCATCGCCCTCGT
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(b)

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      P3                    P4
GluAx AAACAATACA-------CCA TTGGTTCTTTTTGCGGCG
GluAy AAACAGTAC-CCAAAAGCCA TTGGTCCTCTTTGCGACA
GluBx AAACAATACA-------CCA CTGGTCCTCTTTGCGGCA
GluBy AAACAGTAC-CCAGAAGTCA TTGGTCCTCTTTGCGACA
GluDx AAACAATACATCAGAACTAG TTAGTCCTCTTTGTGGCG
GluDy AAACAATAC-CCAGAAGCCA CTGGTCCTCTTTGCGGCA
```

(c)

```
      6 1 1 1 1 1 1 2 2 2 3 3 3 3 3
      0 2 3 4 4 6 8 0 1 3 0 1 1 2 2
        9 3 1 2 1 5 7 3 5 2 0 3 0 5
TAE2  C T T G A T T G T C G A G C C
TAE1  . . . . . C . . . . . . . . .
T3    . . . A . . C . . . . . . . .
T4    . . . A . . C . . . . T . . .
T5    . . . A . . C . . . . . . . T
T6    T . . . . . C . . . . . . . .
T7    . . . . . C C . . . . . . . .
AE3   . . . . . C . . . . . . . T .
AE4   . . G . . . C . . . . . . T .
AE5   . . . . . C A . . . . . T .
AE6   . . . . . C . . . A . . T .
AE7   . . . . . C . A . . . . . .
AE8   . G . . . . C . . . . . A . .
AE9   . . . . G . C . . G . . . . .
```

Fig. 1 Primers and DNA sequences. **a** Positions of the primers P1, P2, P3 and P4 in the *GluDy* promoter region. The sequence is that of allele TAE2. The primer positions are *underlined*, and the TATA box and initiation codon are *double underlined*. The numbering begins at position 1 of primer P3. **b** Alignment of allele sequences from different loci in the P3 and P4 priming regions. Sequences are taken from EMBL accessions M22208 (*GluAx*), X03042 (*GluAy*), X13927 (*GluBx*), X61026 (*GluBy*) and X12928 (*GluDx*) and our results (*GluDy*). *Dashes* indicate indels. **c** Sequences of the amplicons for each of the 14 *GluDy* alleles identified in this study. Only polymorphic positions are shown, numbered from position 1 of primer P3, as in part (**a**). *Dots* indicate identities with the TAE2 sequence. Accession numbers for sequences reported here are DQ233202–DQ233217

P1 and P2. Cycling parameters were: 3 min at 94°C; followed by 29 cycles of 2 min at 55°C, 2 min at 72°C, 1 min at 94°C; followed by 2 min at 55°C and 10 min at 72°C. PCR products were separated by gel electrophoresis in a 1.3% agarose gel and the 442 bp band excised. DNA was extracted using the QIAquick Gel Extraction kit (Qiagen) and eluted in 30 µl of extraction buffer. Nested PCR was performed using 1 µl of a 1:100 dilution of the first round product and 200 ng each of primers P3 and P4. Cycling parameters were as above except that 23 cycles were used and the annealing temperature was 49.5°C. Following the nested PCR, the 346 bp band was purified as before and sequenced using P3 as the primer. Sequences were aligned using CLUSTAL X and a distance matrix created using DNADIST of PHYLIP version 3.6b (Felsenstein 2004) and the assumptions of the Kimura 2-parameter model with a transition:transversion ratio of 2:1 (Kimura 1980). Neighbour-joining trees were constructed and viewed using treeview. Bootstrap analysis was performed using SEQBOOT (100 replications). Networks were constructed as described by Allaby and Brown (2001). Nucleotide diversity (π) was calculated using DnaSP version 3.53 (Rozas and Rozas 1999) based on equation 10.5 of Nei (1987).

Results

The 346 bp amplicon spanned part of the *GluDy* locus from positions −315 to +30 relative to the start of the open reading frame. Previous work has shown that the terminal regions of this amplicon have constant sequences in different *Ae. tauschii* and *T. aestivum* accessions (R.J. Giles, unpublished results). For this reason, and because of the difficulty in obtaining accurate sequence information from the ends of a PCR amplicon, sequence comparisons were limited to a 284 bp internal segment (starting at position 56 in Fig. 1a). Sequences were obtained from 100 accessions of *Ae. tauschii* and 169 of *T. aestivum*. Fourteen different *GluDy* alleles were identified on the basis of the sequence variations shown in Fig. 1c. Nine alleles were present in the *Ae. tauschii* accessions and 7 in *T. aestivum* (Table 1). Two alleles (which we call TAE1 and TAE2) were present in both species; the other 7 alleles from *Ae. tauschii* (AE3–AE9) and 5 from *T. aestivum* (T3–T7) were unique to their respective species. Among the *Ae. tauschii* accessions were 17 that were identified as either ssp. *tauschii* or ssp. *strangulata*. Other than the presence of AE3 in ssp. *tauschii* but not ssp. *strangulata*, there were no clear distinctions between the *GluDy* allele identities of these groups of plants. Allele distributions in the *T. aestivum* subspecies were non-uniform. Six of the 7 *T. aestivum* alleles were present in the 116 ssp. *aestivum* accessions that were tested (the absent allele being T6), and alleles TAE2, T3 and T6 were present in the 17 ssp. *compactum* landraces. The 19 ssp. *spelta* and the 9 ssp. *macha* accessions also displayed variability, but each of the 7 ssp. *sphaerococcum* accessions possessed allele TAE2, as did the 11 ssp. *spelta* accessions from Europe. These data are summarised in Table 1 and nucleotide diversities within the species and subspecies are shown in Table 2. The nucleotide diversity within *Ae. tauschii* was approximately twice that displayed by the *T. aestivum* landraces, with or without the subspecies other than ssp. *aestivum* included in the analysis.

The relationships between the alleles were studied by network construction and by neighbour-joining analysis. The network (Fig. 2) located the shared allele TAE1 at a principal node, from which all other alleles radiate with a star-like phylogeny. The second shared allele, TAE2, is directly linked to the TAE1 node, but has no direct link with any other *T. aestivum* or *Ae. tauschii* allele. The abundant wheat allele, T3, also has a direct link to TAE1. The network suggested that recombination between the 9 *Ae. tauschii* alleles had not occurred, indicating that phylogenetic tree-building is a valid means of examining their evolutionary relationships. Neighbour-joining with various outgroups was therefore performed and in each case AE8 was identified as the ancestral lineage. In the example shown in Fig. 3, with *GluSy* of *Ae. speltoides* as the out-group, a bootstrapping value of 100% gives high support for the spilt between AE8 and

Table 1 *GluDy* alleles in *Ae. tauschii* and *T. aestivum* accessions

Species	Number of accessions	Number of alleles													
		TAE1	TAE2	T3	T4	T5	T6	T7	AE3	AE4	AE5	AE6	AE7	AE8	AE9
All *Ae. tauschii*	100	19	19	–	–	–	–	–	49	1	1	3	1	2	5
ssp. *tauschii*	9	2	2	–	–	–	–	–	4	–	–	–	–	–	1
ssp. *strangulata*	8	4	3	–	–	–	–	–	–	–	–	–	–	–	1
All *T. aestivum*	169	7	134	24	1	1	1	1	–	–	–	–	–	–	–
T. aestivum ssp. *aestivum*	116	6	88	19	1	1	–	1	–	–	–	–	–	–	–
T. aestivum ssp. *spelta*	19	1	18	–	–	–	–	–	–	–	–	–	–	–	–
European *spelta*	11	–	11	–	–	–	–	–	–	–	–	–	–	–	–
Asian *spelta*	8	1	7	–	–	–	–	–	–	–	–	–	–	–	–
T. aestivum ssp. *compactum*	17	–	13	3	–	–	1	–	–	–	–	–	–	–	–
T. aestivum ssp. *macha*	9	–	7	2	–	–	–	–	–	–	–	–	–	–	–
T. aestivum ssp. *sphaerococcum*	7	–	7	–	–	–	–	–	–	–	–	–	–	–	–
T. aestivum ssp. *vavilovi*	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–

Table 2 Nucleotide diversity at the *GluDy* locus within species and subspecies

Group	Number of accessions	Nucleotide diversity (π)[a]
All *Ae. taushii*	100	0.00423
All *T. aestivum*	160[b]	0.00218
T. aestivum ssp. *aestivum*	107[b]	0.00246
T. aestivum ssp. *spelta* (Europe)	11	0
T. aestivum ssp. *spelta* (Asia)	8	0.0088
T. aestivum ssp. *compactum*	17	0.00285
T. aestivum ssp. *macha*	9	0.00274
T. aestivum ssp. *sphaerococcum*	7	0
T. aestivum ssp. *vavilovi*	1	0

[a]Calculated from the variability of the 284 bp region of the *GluDy* promoter region
[b]Excludes the nine cultivars of *T. aestivum* ssp. *aestivum*

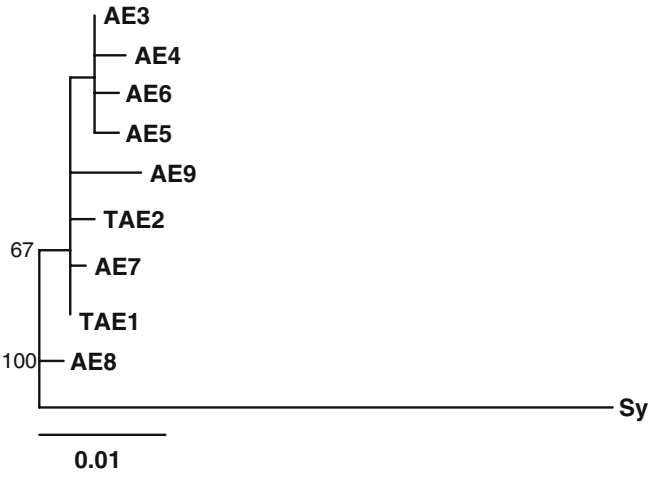

Fig. 3 Neighbour-joining tree showing the relationships between the nine *GluDy* alleles found in *Ae. tauschii*, based on the sequence variations displayed by the promoter region amplicons. The same relationships were obtained when *GluAy* was used as the outgroup

the other alleles. The branching order indicates that TAE1 is most closely related to AE8 (bootstrap 67%), although the rest of the tree is poorly supported.

The *T. aestivum* accessions that were studied included ones with the two most common *GluD1* haplotypes in modern varieties, *GluD1a* and *GluD1d* (Payne and Lawrence 1983). The results for these showed that allele TAE2 corresponds to subunit Dy12 of the *GluD1a* haplotype and T3 corresponds to subunit Dy10 of *GluD1d* (Table 3). The absence of allele T3 in *Ae. tauschii* and the position of this allele in the network suggest that Dy10 of the *GluD1d* haplotype evolved in *T. aestivum* from a TAE1 hexaploid parent. This hypothesis is supported by the allele designations of two *Ae. tauschii* accessions, KU20-10 and KU2090, both of which possess a counterpart of the wheat Dy10 referred to as 10^t (or T5; Dvorak et al. 1998b). Both these accessions possess allele TAE1 and therefore sit at the shared ancestral node in the phylogenetic network. Other *T. aestivum* accessions possessing rare *GluD1* haplotypes were also examined (Table 3). The results for cultivars Hobbit, Champlein, Danchi and 1190377 were as expected, each possessing Dy12 and allele TAE2. Accession 1190421 has allele T7, which is derived from TAE1 (Fig. 2), consistent with its possession of Dy10. However, cultivar Flinor, which has the same haplotype as 1190421, has allele TAE2. Accessions 1190073 and 1190078 possess subunit 2 at the *Dx* locus and a null

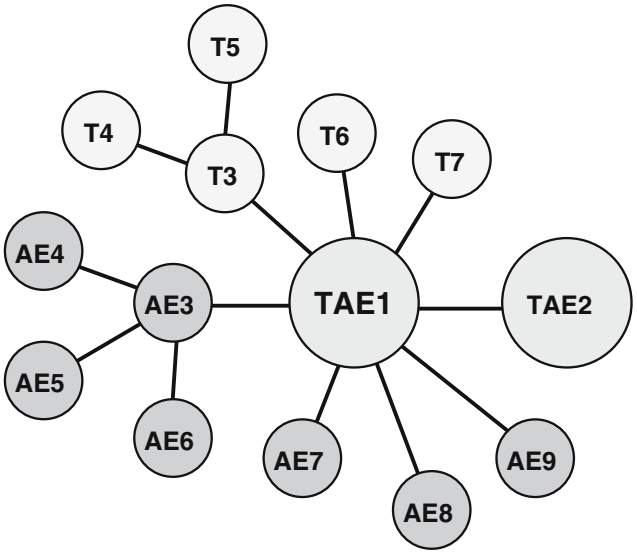

Fig. 2 Network showing the relationships between the 14 *GluDy* alleles based on the sequence variations displayed by the promoter region amplicons. Alleles only found in *T. aestivum* have the prefix T, those only present in *Ae. tauschii* are designated AE, and the two alleles present in both species are denoted TAE

Table 3 *GluDy* allele designations for accessions with known haplotypes

Accession	Glutenin D1 subunits (*Dx* + *Dy*)	Haplotype	*GluDy* allele
T. aestivum cv Chinese Spring	2 + 12	*GluD1a*[a]	TAE2
T. aestivum cv Norin 10	2 + 12[b]	*GluD1a*	TAE2
T. aestivum cv Hobbit	3 + 12	*GluD1b*[a]	TAE2
T. aestivum cv Champlein	4 + 12	*GluD1c*[a]	TAE2
T. aestivum cv Hope	5 + 10	*GluD1d*[a]	T3
T. aestivum cv Neepawa	5 + 10[c]	*GluD1d*	T3
T. aestivum cv Sicco	5 + 10[d]	*GluD1d*	T3
T. aestivum cv Flinor	2 + 10	*GluD1e*[a]	TAE2
T. aestivum cv Danchi	2.2 + 12	*GluD1f*[a]	TAE2
T. aestivum 1190073	2 + null[e]	–	TAE2
T. aestivum 1190078	2 + null[e]	–	TAE2
T. aestivum 1190377	4 + 12[e]	*GluD1c*	TAE2
T. aestivum 1190421	2 + 10[e]	*GluD1e*	T7
Ae. tauschii KU20–10 ssp. *tauschii* var. *meyeri*	5 + $10^{t,f}$	–	TAE1
Ae. tauschii KU2090 ssp. *strangulata*	5 + $10^{t,f}$	–	TAE1

[a]Payne and Lawrence (1983)
[b]Nakamura (1999)
[c]Ahmad (2000)
[d]Popineau et al. (1994)
[e]S. Reader (unpublished results)
[f]Dvorak et al. (1998b)

allele at *Dy*. In both cases the null allele was typed as TAE2, suggesting that the non-expressed subunit is a variant of Dy12.

Approximate but not accurate information was available regarding the collection sites for the *Ae. tauschii* and *T. aestivum* accessions that were studied. For biogeographical analysis, the accessions were therefore grouped into geographical regions, representing as far as possible genuine biogeographical zones for each species, and arranged such that a similar number of accessions could be placed into each region. For *Ae. tauschii*, seven regions were chosen, similar to those used in previous studies (e.g. Dvorak et al. 1998a); these were Turkey, Syria, GRAD (Georgia, Russia, Armenia, Daghestan), CTK (China, Tajikistan, Kazakhstan), Azerbaijan, Iran, and TA (Turkmenistan, Afghanistan). Seven zones were also chosen for *T. aestivum*: TIS (Turkey, Iraq, Syria), Transcaucasia (Russia, Ukraine, Armenia, Daghestan), CMN (China, Mongolia, Nepal), Iran, TUT (Turkmenistan, Uzbekistan, Tajikistan), API (Afghanistan, Pakistan, India), and Europe. Maps showing the geographical distributions of the *GluDy* alleles for *Ae. tauschii* and *T. aestivum* landraces, the latter with and without inclusion of the non-*aestivum* subspecies, are shown in Fig. 4. Nucleotide diversities for these geographical populations are shown in Tables 4 and 5.

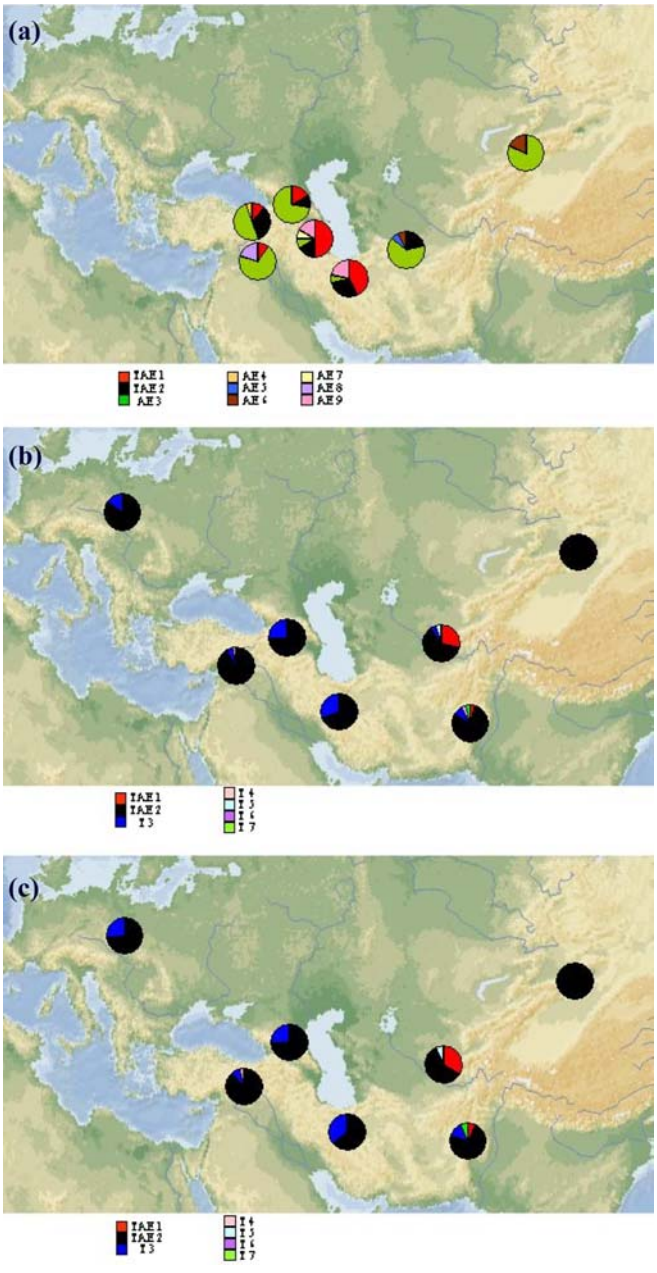

Fig. 4 Geographical distributions of the *GluDy* alleles for **a** *Ae. tauschii*, **b** *T. aestivum* landraces with inclusion of the non-*aestivum* subspecies, and **c** *T. aestivum* landraces without inclusion of the non-*aestivum* subspecies

Discussion

Evolution of the GluDy locus in hexaploid wheats

The *Ae. tauschii* and *T. aestivum* accessions that we studied possessed a total of 14 *GluDy* alleles, two of which were shared between the species. The existence of two shared alleles suggests that there were at least two independent origins of hexaploid wheat, as also indicated by electrophoresis of glutenin proteins (Lagudah and Halloran 1988), RFLP analysis (Dvorak et al. 1998a), PCR of sequence tagged sites (Talbert et al. 1998), microsatellite analysis (Lelley et al. 2000), and examination of the *Xwye838* and *Gss* loci (Caldwell et al. 2004). An alternative explanation, that the ancestral *Ae. tauschii* was heterozygous at the *GluDy* locus, thereby contributing both TAE1 and TAE2 via a single polyploidisation, is unlikely as *Ae. tauschii* is predominantly an inbreeder and heterozygotes are rare. Lubbers et al. (1991), for example, examined 25 loci in 102 accessions of *Ae. tauschii*, and found every locus to be homozygous in every plant.

The possibility that the C to T transition at position 185 of our sequence alignment (Fig. 1c), which distinguishes TAE1 from TAE2, occurred in parallel in *Ae. tauschii* and *T. aestivum* is also unlikely. The results given in Table 3 show that the Dy12 subunit of haplotype *GluD1a* is coded by TAE2 and the Dy10 subunit of *GluD1d* by T3. TAE2 and T3 are distinguished by two SNPs in the amplicon that we studied (Fig. 1c), but the coding sequences for subunits Dy12 and Dy10 of the *GluD1a* and *GluD1d* haplotypes in *T. aestivum* also have a number of sequence differences, including two indels totalling 40 bp (Mackie et al. 1996b). The complete allele sequences for TAE2 and T3 are therefore significantly different. Equivalents of the *GluD1a* and *GluD1d* haplotypes are also recognised in *Ae. tauschii* (Lagudah and Halloran 1988). Our analysis shows that in *Ae. tauschii* subunit $Dy10^t$ is specified by TAE1, and that this allele is also present in a small number of wheats. Although subunits Dy10 and $Dy10^t$ are not identical, having different surface hydrophobicities (Mackie et al.

Table 4 Nucleotide diversity (π) at the *GluDy* locus for *Ae. tauschii* accessions grouped into geographical regions

Geograpahical region	Number	π^a
Turkey	19	0.00389
Syria	10	0.00415
Georgia, Russia, Armenia, Daghestan	18	0.00223
China, Tajikistan, Kazakhstan	11	0.00115
Azerbaijan	12	0.00437
Iran	14	0.00460
Turkmenistan, Afghanistan	14	0.00356

[a]Calculated from the variability of the 284 bp region of the *GluDy* promoter region

1996a), the size similarities suggest that they are closely related, the most likely explanation being that Dy10 is a modified version of $Dy10^t$ that arose after the transfer of allele TAE1 into *T. aestivum*. This close relationship between T3 and TAE1 (with both specifying the same sized protein) makes it highly likely that they have the same indels within the coding region when compared to the coding region of TAE2. If this argument is correct, then the SNP that separates TAE1 and TAE2 in the amplicon that we studied is accompanied by more substantial sequence differences downstream from this amplicon, making it extremely unlikely that parallel evolution is responsible for the presence of TAE1 and TAE2 in both *Ae. tauschii* and *T. aestivum*. In short, there were at least two separate origins of hexaploid wheats.

As described above, from examination of the five accessions with the *GluD1a* or *GluD1d* haplotypes it was apparent that T3 codes for the Dy10 subunit and TAE2 codes for Dy12. These identifications were confirmed by consideration of eight other *T. aestivum* accessions with known haplotypes (Table 3). The four accessions with haplotypes *GluD1b*, *GluD1c* or *GluD1f*, each of which is associated with subunit Dy12, were shown to possess allele TAE2, as expected. The two accessions that possess Dx2 and a null Dy protein (1190073 and 1190078), also had the predicted TAE2 allele. Just 2 of these 13 accessions, Flinor and 1190421, both with the rare haplotype *GluD1e* and subunit composition 2 + 10, gave anomalous results. The presence of Dy10 in these accessions suggests that their *GluDy* allele should be T3, but Flinor had TAE2 and 1190421 had T7. Payne and Lawrence (1983) have suggested that *GluD1e* could have arisen through recombination between *GluD1a* and *GluD1d*. Conceivably such an event could attach the promoter region associated with *GluD1a* (i.e. TAE2) to a coding region associated with *GluD1d* (i.e. subunit 10), but a scheme to achieve this is difficult to construct bearing in mind that *GluDy* is upstream of *GluDx*. Alternatively, the Dy10 subunit possessed by Flinor could have arisen via one or more deletions in the coding region for a Dy12 subunit, resulting in a protein that has a similar size to the Dy10 subunit coded by T3 but is specified by TAE2. It is worth noting that sequence elimination is the major and immediate response of the wheat genome to allopolyploidy (Shaked et al. 2001). In contrast, the presence of T7 in 1190421 is more easily explained, as T7 is derived from TAE1 (Fig. 2) and hence might be expected to code for a Dy10-type subunit. These interpretations of the *GluD1e* results have two implications. First, this haplotype is heterozygous as Flinor and 1190421 have different origins, Second, the presence in *Ae. tauschii* of the 2 + 10 combination of subunits that characterises *GluD1e* (Lagudah and Halloran 1988) raises the possibility that the T7 allele, which we detected in no accession other than 1190421, derives from a third hexaploidisation. Further analysis of additional accessions and longer amplicons is needed to settle these points.

Table 5 Nucleotide diversity (π) at the *GluDy* locus for *T. aestivum* landraces grouped into geographical regions

Geographical region	All *T. aestivum*		*T. aestivum* ssp. *aestivum*	
	Number	π^a	Number	π^a
Turkey, Iraq, Syria (TIS)	33	0.00141	25	0.00183
Transcaucasia	19	0.00288	8	0.00302
China, Mongolia, Nepal (CMN)	14	0	12	0
Iran	16	0.00323	14	0.00348
Turkmenistan, Uzbekistan, Tajikistan (TUT)	21	0.00272	15	0.00275
Afghanistan, Pakistan, India (API)	25	0.00228	15	0.00282
Europe	26	0.00191	15	0.00295

[a]Calculated from the variability of the 284 bp region of the *GluDy* promoter region

Triticum aestivum possesses at least 5 unique *GluDy* alleles, compared to just 7 in *Ae. tauschii*, and within the amplicon we studied has approximately half the nucleotide diversity of *Ae. tauschii*. A relatively high nucleotide diversity for *T. aestivum*, though still lower than that of *Ae. tauschii*, has also been noted by Talbert et al. (1998), who studied a 527 bp region of the D genome, and by Huang et al. (2002b) who compared microsatellite loci. This is despite hexaploid wheat being the product of only 10,000 years of evolution, preceded by a genetic bottleneck, whereas *Ae. tauschii* has an evolutionary history of 2.5–4.5 million years (Huang et al. 2002a). Allopolyploidisation is known to be associated with rapid and dramatic changes in the constituent genomes, including epigenetic changes such as DNA methylation (Liu et al. 1998; Liu and Wendel 2002) which, under some circumstances, can lead to sequence change, such as the C to T transition that may occur when a methylated C residue undergoes deamination (Razin and Riggs 1980). Reversible gene silencing of the *Glu1* loci of *T. aestivum*, which might involve DNA methylation, has been reported by Galili and Feldman (1983, 1984), and hence a mechanism for driving sequence change at this locus may exist.

Geographical structure of the *GluDy* genepool in *Ae. tauschii*

Neighbour-joining analysis identified AE8 as the ancestor of the 14 *GluDy* alleles present in *Ae. tauschii* and *T. aestivum* (Fig. 3). AE8 is a rare allele, present in only two accessions, both from northern Syria (Fig. 4a). According to Hammer (1980), the centre of origin of the *Aegilops* genus is Transcaucasia and the most primitive species have distribution zones close to this centre (e.g. *Ae. speltoides* is restricted to Turkey). Additionally, van Slageran (1994) reported that the D genome *Aegilops* populations from the central part of the Fertile Crescent display the greatest morphological diversity. Allowing for genetic erosion and changing biogeography, the presence today of the ancestral AE8 allele in northern Syria is therefore not incompatible with other data. From its point of origin *Ae. tauschii* spread in a predominantly eastward direction and today the centre of its distribution lies in Azerbaijan and on the southern shore of the Caspian Sea (van Slageran 1994). In agreement with this model, and the results of other genetic studies (e.g. Lubbers et al. 1991), we found that populations of *Ae. tauschii* in Azerbaijan and Iran had the greatest *GluDy* diversities (Table 4).

AE3 is the commonest *GluDy* allele in *Ae. tauschii* (Table 1), and also has a star-like phylogeny (Fig. 2), consistent with an ancient origin in a region where its parental TAE1 allele was already present. The relative scarcity of AE3 in Azerbaijan and Iran (Fig. 4a) may imply that this origin was further west. However, AE3 has spread to all the other regions and in the eastern-most populations it and its daughters (AE4–AE6) predominate. The position of TAE2 in the network (Fig. 2) suggests that this allele may have evolved relatively recently, but the biogeography gives no indication of where this might have been, TAE2 being common throughout the arc spreading from Turkey in the west to Turkmenistan and Afghanistan in the east.

The traditional classification of *Ae. tauschii* splits the species into two main subspecies, ssp. *strangulata* and ssp. *tauschii*, based primarily on differences in spike morphology (Jaaska 1981). Subspecies *tauschii* is further split into a further three variants, var. *tauschii* (*typica*), var. *anathera*, and var. *meyeri*. Until recently it was thought that ssp. *strangulata* had two distinct distribution zones, one in Transcaucasia and one in Southwest Caspian Iran. In contrast ssp. *tauschii* was thought to be widespread, occurring throughout the geographical range of the species. Studies using ribosomal DNA (Kim et al. 1992) and RFLPs (Lubbers et al. 1991; Tsunewaki et al. 1991; Dvorak et al. 1998a) have shown that the classification using morphological criteria is inadequate. Dvorak et al. (1998a) identified just two genetic groupings of *Ae. tauschii*, which they call the *tauschii* and *strangulata* genepools, the latter including some plants that morphologically belong to ssp. *tauschii*. Among the accessions that we studied, subspecies identities were known for only a few plants. There was not, however, any clear split of *GluDy* alleles between these subspecies, except that 4 of the 9 ssp. *tauschii* specimens were typed as AE3 whereas this allele was unrepresented among the 8 ssp. *strangulata* accessions (Table 1). A coincidence between AE3 and the *tauschii* genepool becomes more apparent when the biogeography is considered (Fig. 4a), AE3 being most frequent in those areas that Dvorak et al. (1998a) associate with the *tauschii* genepool (i.e. Syria, Turkey and east of Iran). Our results therefore provide further evidence that the morphological classification of plant species may have little association with the actual ancestral relationships between individuals.

The nucleotide diversity data (Table 4) show that, outside of the species' probable centre of diversity in Azerbaijan/Iran, the greatest diversity within *Ae. tauschii* occurs in Syria and Turkey. Again, bearing in mind the limitations of these data, the relatively high π values for these two regions appear inconsistent with a recent adventive spread as suggested by van Zeist (1976) and Zohary and Hopf (2000), especially as the nucleotide diversity in the Far East, whose populations are also attributed to a recent expansion (van Slageran 1994), is much lower. The diversity in Syria and Turkey, along with the presence of the three major lineages (including the ancestral allele AE8), indicates that these populations are probably ancient, and that *Ae. tauschii* was therefore present in this part of the Fertile Crescent before the origins of agriculture.

The *GluDy* genepool in *T. aestivum*

As described above, our results indicate that hexaploid wheats have at least two separate origins, one giving rise to the lineage possessing the TAE1 allele and its derivatives, and the other giving rise to the lineage with TAE2. The biogeographical data shown in Fig. 4b, c indicate that these two lineages are unlikely to have originated in the same place, as the modern day distributions are quite different, TAE2 being widespread throughout Eurasia whereas TAE1 is limited to central and southern Asia. TAE2 is present in 80% of the *T. aestivum* landrace population including all six subspecies (Table 1) which, together with its widespread distribution, suggests that the TAE2 hexaploid lineage predates the TAE1 lineage. The origin of farming in the western arm of the Fertile Crescent means that the first contact between cultivated *T. turgidum* and wild *Ae. tauschii* probably occurred in a region at the western edge of the distribution zone of *Ae. tauschii*. This includes the population in southeast Turkey, which we believe to be ancient, and which has a high frequency of TAE2 (Fig. 4a). An origin of hexaploid wheat in Turkey is consistent with its first appearance in the archaeological record at the early farming sites of Cafer Höyük, Can Hasan III and Çatalhöyük in Turkey and Abu Hureyra in Syria (Hillman 1978; Moore et al. 2000; de Moulins 1993, 2000; Fairbairn et al. 2002). Our interpretation therefore resolves the conflict between previous genetic

studies, which placed the hexaploidisation event(s) either in Transcaucasia or the south Caspian region close to the current centre of diversity of *Ae. tauschii*, and the archaeological evidence which provides no indication of hexaploid wheat in these regions until some 1,300 years after its first recorded appearances in Turkey and Syria.

The absence of the TAE1 allele in *Ae. tauschii* populations from Afghanistan, Tajikistan, Turkmenistan and Uzbekistan (Fig. 4a) suggests that the TAE1 hexaploid lineage did not originate in the region in which it is now found. It is more likely that this lineage arose in neighbouring Iran, where TAE1 is common in *Ae. tauschii*, with the hexaploid lineage then following a west to east expansion into its current distribution zone (Fig. 4b, c). Iran has the highest frequency of the *T. aestivum* allele T3, which is derived from TAE1, supporting an Iranian origin for the TAE1 lineage if one assumes that T3 evolved from TAE1 soon after formation of the hexaploid as a result of genomic changes accompanying polyploidisation (see above). The current distribution of T3 is distinct from that of TAE1, suggesting that the T3 population did not follow the same expansion pattern as its parent. This could be due to the effect of random genetic drift in what would have initially been relatively small populations of hexaploid wheat. However, the discovery that T3 corresponds with the *GluD1d* haplotype, which has superior baking qualities when compared to other D genome haplotypes (Payne and Lawrence 1983; Dong et al. 1991), indicates that positive selection could have resulted in T3 being more broadly distributed than its TAE1 parent.

The subspecies designations of the *T. aestivum* accessions appear to have little influence on the identities of the *GluDy* alleles present in each accession (Table 1). The conclusion that geographical location rather than subspecies is the major influence on genotype was previously reached when RAPD analysis was used to compare ssp. *spelta* and ssp. *macha* (Cao et al. 1998) and when comparisons were made between the microsatellite genotypes of *T. aestivum* varieties (Huang et al. 2002b). A link between geography and genotype is most apparent among European spelt wheats, as all 11 of these accessions possessed the TAE2 allele (Table 1), which is also the most common allele in hexaploid wheats from Europe, particularly western Europe. This observation is consistent with a central or western European origin for these spelt wheats. The low genetic diversity present at the *GluDy* locus in Asian spelt wheats (Table 1) is inconsistent with the proposal that Asian spelt is the ancestral form of all hexaploids. *T. aestivum* ssp. *compactum* showed the highest variability at the *GluDy* locus compared with other subspecies, but most of the ssp. *compactum* accessions originated from central Asia, where overall variability of *T. aestivum* is highest, which could have influenced this result. Overall, the results of this study make it unlikely that any of the six subspecies have independent origins.

The domestication of wheat involved changes to several genes, one of the most important of these being Q which confers the free threshing character and influences several important traits (Faris et al. 2003). As yet it is unknown in which species the changes to Q occurred. The likely origin of hexaploid wheat some 1,000 years earlier than previously assumed indicates that there are many unanswered questions regarding the domestication and evolution of our most important cereal crops.

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