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Multiple origins of allopolyploid *Aegilops triuncialis*

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Abstract Polyploidization is a key component of plant evolution. The number of independent origins of polyploid species traditionally has been underestimated. The objective of this study was to ascertain the number of origins of a tetraploid *Aegilops* species. We screened 84 primer sets to identify genome-specific primer sets for the tetraploid wheat relative [*Aegilops triuncialis* (UUCC genome)] and its diploid progenitors [*Ae. umbellulata* (UU genome) and *Ae. caudata* (CC genome)]. Primer sets G12 and G43 were U genome-specific and D21 was a C genome-specific primer. DNA sequence comparison of the G43 locus was used to estimate the number of polyploidization events in the formation of *Ae. triuncialis*. Parsimony analysis of G43 data revealed at least two independent formations of *Ae. triuncialis*. In the chloroplast hotspot region, located between genes *rbc*L and *pet*A, sequence analysis suggested that at least three polyploidization origins might have occurred independently. *Ae. triuncialis* appears to be a tetraploid derived from multiple origins with minimal genome change after its formation.

Keywords Aegilops · Polyploidization · Wheat relatives · Allopolyploid

Introduction

Within the plant kingdom, polyploidization is a powerful process leading to speciation as well as providing genetic variation. In general, 70% of angiosperms have undergone polyploidization at least once (Masterson 1994). Polyploid species tend to be more widely distributed and found in more extreme habitats than their diploid ancestors (Soltis and Soltis 2000). Understanding the number of polyploidization events that have occurred in the formation of a given species, and the consequences of such events, has been a major challenge. The traditional point of view was that polyploidization events were rare because less variability within polyploid species was observed than within the diploid relatives (Stebbins 1971). However, more recently, recurrent formation of polyploids has been demonstrated. Over 30 examples of allotetraploid species have been shown to have multiple origins to date (Soltis and Soltis 1999). Hexaploid wheat formed at least twice from its diploid progenitor, *Aegilops tauschii* (DD genome) (Dvorak et al. 1998; Talbert et al. 1998).

Allopolyploids receive their chromosome sets from different species, unlike autopolyploids that receive multiple sets of chromosomes from one species. The prevalence of allopolyploid formation and the degree of genetic separation between allopolyploids and their progenitors have important consequences for the accumulation of genetic variability within an allopolyploid. *Aegilops triuncialis* (UUCC genome) is an allotetraploid that resulted from reciprocal crosses of the diploids *Ae. umbellulata* (UU genome) and *Ae. caudata* (CC genome) (Wang et al. 1997). *Aegilops triuncialis* is the most widespread *Aegilops* species in the world. This grass is distributed between altitudes of 300 m and 1000 m and has become a troublesome weed on U.S. rangelands (Watanabe and Kawahara 1999). Its successful adaptation may result from the genetic attributes of polyploids, such as high genetic variability and the evolution of new gene functions (Soltis and Soltis 2000).

Molecular approaches including restriction fragment analysis, comparative sequencing and various polymerase chain reaction (PCR)-based techniques are now available to uncover recurrent origins of allopolyploids. Within a plant cell, three different types of DNA are found: nuclear, chloroplast (cp) and mitochondria (mt) DNA. Most current molecular data have come from the chloroplast, the highly repetitive sequences of ribosomal

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RNA (rRNA) and low-copy genes (Soltis et al. 1998). Genome-specific primers may be used to analyze the biparentally inherited nuclear genome of an allopolyploid. The aim is to lessen ambiguous results by demonstrating direct inheritance of an allele from a specific ancestral genome. Small et al. (1999) derived genome specific primers for the *AdhA* locus in tetraploid cotton by cloning and sequencing homoeologous products from each subgenome. These primers were used to assess nucleotide diversity within tetraploid cotton. Talbert et al. (1998) used nullitetrasomic wheat stocks to identify a D genome-specific primer, A1, in wheat. Sequence comparison of A1 amplicons in wheat and the D genome diploid progenitor, *Ae. tauschii*, indicated that hexaploid wheat was formed at least twice. Chee et al. (1995) used primer sets specific for the U and M genomes of *Aegilops* to show that tetraploid UUMM accessions shared restriction site polymorphisms with both diploid progenitors, indicating multiple origins of the polyploid.

Because of the size and slow mutation rate of the chloroplast genome, it has several advantages for taxonomic and evolutionary studies. The genome is quite small, approximately 120–200 kb. In Chinese Spring wheat, the chloroplast genome is 134,540 bp long (Ogihara and Tsunewaki 2000). CpDNA is usually maternally inherited in plants. The genome is relatively conserved during its evolution throughout plant species. The chloroplast genome evolves four to five times slower than the nuclear genome and three times faster than that of mitochondria (Page and Holmes 1998). Because different regions of DNA evolved at different rates, some parts of the cpDNA might be appropriate to resolve relationships at different taxonomic levels (Soltis et al. 1998). Provan et al. (2001) reviewed the use of noncoding regions and chloroplast microsatellites for intraspecific and interspecific studies in *Glycine*, rice, and wheat (Powell et al. 1996; Ishi and McCouch 2000; Ishi et al. 2001). The chloroplast gene encoding the large subunit of ribulose-1, 5-bisphosphatecarboxylase/oxygenase (*rbc*L) was used extensively in molecular systematic studies among angiosperms (Chase et al. 1993). Other chloroplast genes such as *atp*B, *ndh*F and *mat*K, were also utilized (Judd et al. 1999).

All previous studies have confirmed a maternal lineage of chloroplast inheritance among *Triticum* and *Aegilops* species (Ogihara and Tsunewaki 1982; Murai and Tsunewaki 1986; Tsunewaki 1993; Wang et al. 1997). Ogihara and Tsunewaki (1988) defined 16 chloroplast types in *Triticum* and *Aegilops* species based on restriction fragment length polymorphism (RFLP). Their results showed that six of fourteen insertion/deletion mutations (indels) were located between the genes *rbc*L and *pet*A. The region was designated as a hotspot and not only contained many direct and inverted repeats near the indel region but also was AT-rich (Ogihara et al. 1992). They suggested that these two characteristics might be responsible for the high mutation rate. Previous studies of alloplasmic wheat lines containing *Ae. caudata, Ae. triuncialis* and synthetic *Ae. triuncialis* cytoplasm had type-2 chloroplast, which included a 300-bp deletion within the hotspot region. Unlike *Ae. caudata, Ae. umbellulata* cytoplasm in an alloplasmic line had type-3 chloroplast lacking this deletion (Ogihara and Tsunewaki 1988). The deletion was one of many structural changes distinguishing *Ae. caudata* and *Ae. umbellulata*. This region seemed promising for differentiating these genomes, although only one alloplasmic line per species was analyzed. Further study using nucleotide sequence comparison revealed that nucleotides at the intergenic regions diverged ten times faster than those of coding regions (Ogihara et al. 1991).

Evidence from chloroplast studies of Murai and Tsunewaki (1986) and Wang et al. (1997) demonstrated that *Ae. triuncialis* derived from reciprocal crosses of *Ae. caudata* and *Ae. umbellulata*. Murai and Tsunewaki (1986) revealed that 13 accessions of *Ae. triuncialis* had type-2 chloroplast as did an alloplasmic line with *Ae. caudata* cytoplasm. Eight accessions of *Ae. triuncialis* contained type-3 chloroplast as did an alloplasmic line with *Ae. umbellulata* cytoplasm. The 300-bp deletion within the hotspot region was presumed to be specific to the C genome. Wang et al. (1997) confirmed these results, as well as determining that the two types of *Ae. triuncialis* were formed recently and concurrently.

For this report, we identified genome-specific primers for the U, C and chloroplast genomes, to allow unambiguous genome assessment of products amplified from *Ae. triuncialis*. Since DNA sequence comparison provides the greatest discrimination of evolutionary relationships, sequence data were obtained from DNA segments amplified using these genome-specific primers. Phylogenetic analysis based on DNA sequence data was performed to determine the relationships of the diploid ancestors, *Ae. caudata* and *Ae. umbellulata* and the allotetraploid, *Ae. triuncialis*. Data was evaluated to assess the number of origins of *Ae. triuncialis*.

Materials and methods

Plant materials

Thirty-one accessions of *Aegilops caudata*, 33 accessions of *Ae. umbellulata* and 212 accessions of *Ae. triuncialis* were obtained from USDA National Small Grain Collection, Aberdeen, Idaho, USA. Three accessions of *Ae. caudata*, two accessions of *Ae. umbellulata* and six accessions of *Ae. triuncialis* were requested from the Wheat Genetics Resource, Kansas State University. Four accessions of *Ae. triuncialis* were obtained from the Plant Germplasm Institute, Kyoto University Japan (http://www.shigen.nig.ac.jp/wheat/chloroplast/) (Murai and Tsunewaki 1986). *Aegilops tauschii* accession KU2050 was used as an outgroup species. All plants were grown in the Plant Growth Center at Montana State University, and young leaves were collected for total genomic DNA extraction (Riede and Anderson 1996). A single plant was used from each accession. Genomic DNA was adjusted to approximately 100 ng/µl for use as template DNA in PCR reactions.

PCR primers

A total of 84 primer sets designed from wheat, *Ae. tauschii*, barley and oat (Tragoonrung et al. 1992; Talbert et al. 1994; Erpelding et

al. 1996) were used to screen accessions of *Ae. caudata*, *Ae. umbellulata* and *Ae. triuncialis*. Primers were synthesized by Sigma Genosys, USA. Six primers were designed for each side of the chloroplast hotspot region and tested in all combinations to identify the most robust pair. The Cp6 (U6/R6) primer set was selected to amplify the noncoding region between the *ycf*4 and *cem*A genes within the chloroplast genome of *Triticum* and *Aegilops* species. PCR amplification, digestion and analysis were performed using the protocol of Talbert et al. (1994).

Statistical analysis

A χ^2 goodness-of-fit test was performed to test the nuclear and chloroplast correspondence for G43 and U6/R6 loci within *Ae. triuncialis*. A 2×2 two-way table was used for the χ^2 goodness-of-fit test.

Cloning and DNA sequencing

PCR products were cloned prior to DNA sequencing using the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.) or pGEM-T vector (Promega, Madison, Wis.). Sequencing was done on an ABI377 automated DNA sequencer with the Perkin Elmer BigDye sequencing reaction kit (PE Biosystems, Foster City, Calif.). Sequences were read in both the forward and reverse directions using either the original primer sets or cloning vector primers.

Phylogenetic analysis

Sequences were initially aligned by ALIGN program (Scientific and Educational Software 1989) followed by manual alignment to minimize gaps. The data were analyzed with maximum parsimony as implemented in the computer program PAUP* version 4.0 beta8 (Swofford 1998). Parsimony analysis counteracts sequence errors due to PCR of cloned amplicons since it is necessary that at least two taxa share a nucleotide change for it to be used to construct phylogenetic relationships. The stepwise addition option was used to find the most parsimonious bootstrap trees. Bootstrapping was performed using the full heuristic search option of PAUP* to calculate the robustness of each branch. The analysis was set with the following parameters: 100 bootstrap replicates (Felsenstein 1985) with gaps treated as missing data, tree bisection-reconstruction branch swapping and random sequence addition. All characters were weighted equally. Bootstrap values indicated the percentage of time that resampling yielded the same clade. The goodness-of-fit statistic estimated the reliability of each phylogenetic tree. Consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated (Kluge and Farris 1969; Farris 1989). *Aegilops tauschii* accession KU2050 was used as an outgroup taxon.

Results and discussion

A total of 84 primer sets were screened on at least two accessions each of *Ae. umbellulata, Ae. caudata* and *Ae. triuncialis* in order to identify genome-specific primers. PCR products were amplified at annealing temperatures of either 45 °C (71.43%) or 50 °C (68.65%). All of the primers tested were derived from related species (*Ae. tauschii*, wheat, barley and oat) and may not have amplified or given less repeatable results than primers developed from the target species (Erpelding et al. 1996; Vanichanon et al. 2000). In our results, 46 primer sets amplified all three genomes, and only three primer sets were genome-specific. U genome-specific primers were G12 and G43 and primer set D21 was C genome-specific.

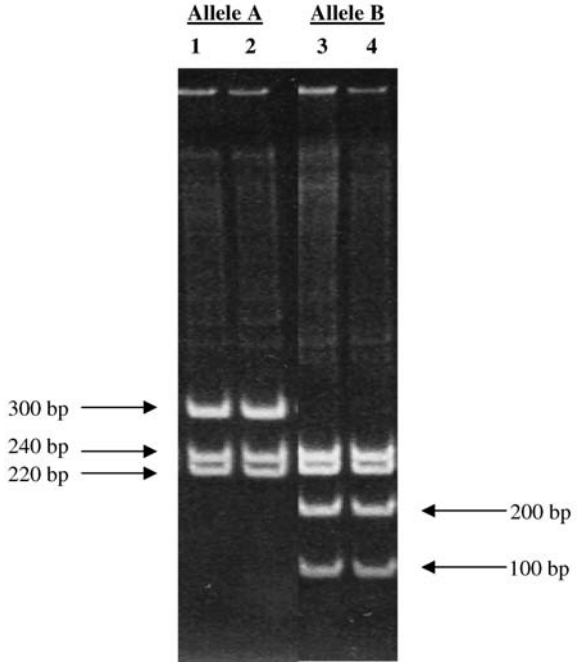

Fig. 1 *Dde*I-digested DNA amplified from *Aegilops umbellulata* and *Ae. triuncialis* using primer G43. *Lanes 1, 2* allele A, *lanes 3, 4* allele B. *Lanes*: *1 Ae. umbellulata* (U05) *2 Ae. triuncialis* (UC10) *3 Ae. umbellulata* (U08) *4 Ae. triuncialis* (UC04)

Only locus G43 showed sufficient sequence polymorphism among accessions for phylogenetic analysis.

Multiple origins of *Ae. triuncialis* inferred using nuclear DNA sequence analysis

Nuclear DNA analysis was chosen to assess multiple origins of tetraploid *Ae. triuncialis*. Genome-specific primers were preferred because they provided unambiguous evidence that a specific *Ae. triuncialis* pattern came from either *Ae. umbellulata* or *Ae. caudata*. Therefore, G43, a U genome-specific primer, was selected for further evaluation (Chee et al. 1995). *Dde*I restriction digestion of the G43 amplicon yielded two banding patterns: allele A (Fig. 1, lanes 1, 2; fragment sizes: 300, 240, 220 bp) and allele B (Fig. 1, lanes 3, 4; fragment sizes: 240, 220, 200, 100 bp). Both alleles were observed in *Ae. umbellulata* and *Ae. triuncialis*. This is evidence that *Ae. triuncialis* inherited two distinct alleles from *Ae. umbellulata*, indicating that at least two distinct polyploidization events occurred.

A single nucleotide change detected by restriction fragment analysis may not be sufficient to determine evolutionary relationships since independent mutations may give rise to the same polymorphism. Because of limited data from the restriction fragment studies, the G43 locus was selected for comparative DNA sequence analysis to determine whether the RFLP was indicative of distinct alleles. We sequenced G43 alleles from ten accessions of *Ae. umbellulata* and eight accessions of *Ae.*

Table 1 List of *Aegilops umbellulata* and *Aegilops triuncialis* accessions used in DNA sequence comparisons of locus G43

Species	Accession	Lab designation
Ae. umbellulata	CIae 66	U01
	PI204546	U02
	PI222762	U03
	PI227339	U04
	PI227436	U05
	PI298907	U07
	PI428569	U08
	PI486256	U09
	PI487247	U10
	PI573515	U14
Ae. triuncialis	PI542322	UC01
	PI542279	UC02
	PI374344	UC04
	PI226501	UC05
	PI219864	UC06
	PI542325	UC07
	PI551178	UC11
	PI574471	UC15

triuncialis (Table 1). A total of 802 bases of DNA sequence were obtained for all alleles. Sequence data confirmed the assumption from restriction analysis that both *Ae. umbellulata* and *Ae. triuncialis* bear the same two distinct alleles. Table 2 tabulates nucleotide differences that distinguish the two alleles. A total of 17 nucleotide changes differentiated allele A from allele B in *Ae. umbellulata* and *Ae. triuncialis*. We assumed that allele A in the *Ae. triuncialis* accessions was inherited from allele A in the *Ae. umbellulata* accessions, either paternally or maternally, and similarly for allele B.

Multiple alignment of G43 sequences revealed 36 parsimony sites and 97 variable sites within ten accessions of *Ae. umbellulata*. There were 28 parsimony sites and 35 variable sites within eight accessions of *Ae. triuncialis*. All 28 parsimony sites and 35 variable sites in *Ae. triuncialis* were also found in *Ae. umbellulata*. Hence, all polymorphisms in the polyploid appear to have been inherited from the diploid progenitor.

The low levels of polymorphism observed and the fact that all the polymorphisms in *Ae. triuncialis* occurred in *Ae. umbellulata* agrees with Liu et al. (2001) who observed that a slow rate of genomic change occurred in synthetic allopolyploid cotton (*Gossypium*) species. They concluded that a rapid rate of genome changes after polyploidization was not the rule. A significant difference between our study and the cotton study was that *Ae. triuncialis* is a natural allopolyploid not a synthetic one. This is evidence that synthetic and naturally occurring allopolyploids can undergo similar levels of genome change after polyploidization. However, synthetic allopolyploid *Brassica*, *Triticum* and *Aegilops* species showed a rapid rate of genomic changes (Song et al. 1995; Liu et al. 1998a, b). From synthetic allopolyploid *Brassica*, Song et al. (1995) concluded that the more closely related the parents, the fewer genomic changes that occurred. Therefore, one possible reason for the apparent slow rate of genomic change in *Ae. triuncialis* may be due to closely related diploid parents (Badaeva et al. 1996).

A PAUP-generated phylogenetic tree (Fig. 2) was developed with a minimal tree length of 86. Goodness-of-fit statistics were calculated. Consistency, retention and rescaled consistency indices were 0.9186, 0.9517

Table 2 Polymorphic nucleotide positions in locus G43. Polymorphic nucleotide position site numbers given above sequences. *Ae. umbellulata* accession U01 is reference sequence for all other accessions. A period denotes nucleotide identity to the reference sequence; gaps are indicated by dashes. Allelic designations are given in the final column. Accession identification is given in Table 1

Accession	Site number																	Allele
	7	17	107	305	306	307	309	320	358	361	365	386	389	410	411	412	413	
U01	T	G	A	T	C	G	C	T	C	C	C	A	A	G	–	–	–	A
U02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
U03	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
U04	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
U05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
U07	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
U08	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
U09	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
U10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
U14	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC01	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
UC04	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC05	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC06	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC07	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B
UC11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
UC15	A	T	G	–	–	–	T	C	T	T	A	G	G	C	T	A	A	B

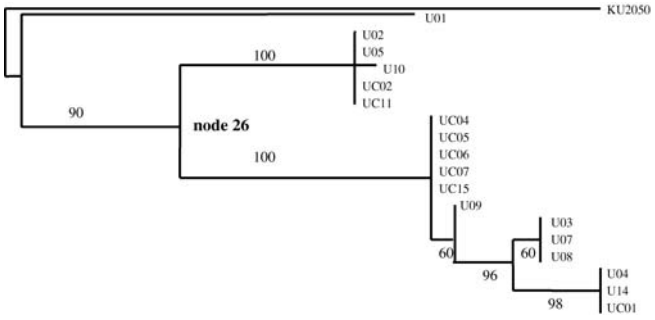

Fig. 2 Maximum parsimony tree of locus G43 derived from heuristic search with a length of 86, CI of 0.9186, RI of 0.9517 and RC of 0.8743. Bootstrap values are given about each node. Accession designations are given in Table 1. *Ae. tauschii* KU2050 was the outgroup taxon

Fig. 3 DNA sequences of intergenic region between *ycf*4 and *cem*A in chloroplast genome from *Ae. umbellulata* (CIae66). The *boxed region* indicates the deletion that occurs in some *Aegilops* chloroplasts. *Bold italicized* nucleotides represent a pair of direct repeats. *Bold double-underlined* nucleotide demonstrates G/T transversion at position 58,329. *Bold single-underlined* nucleotides show additional polymorphisms at positions 58,276 and 58,523 that are associated with the G/T transversion at position 58,329. *Underlined* nucleotides are left and right primers in *ycf*4 and *cem*A genes, respectively

and 0.8743, respectively. At node 26, the split into A- and B-allele clades gave a bootstrap value of 100% of resampling (Fig. 2). The A-allele clade contained both *Ae. umbellulata* and *Ae. triuncialis* accessions, as did the B-allele clade. Thus, all G43 outcomes indicated that polyploidization occurred at least twice.

Multiple origins of *Ae. triuncialis* inferred using cpDNA sequence analysis

We designed six primers on each side of the chloroplast hot spot region between *rbc*L and *pet*A and tested them in all combinations to identify the most reliable pair. Primer set U6/R6 amplified a 600-base intergenic region between *ycf*4 and *cem*A that included the area involved in the 300-bp deletion (Ogihara and Tsunewaki 1988). PCR analysis results showed that 23 *Ae. caudata* accessions had the deletion (nucleotides inside the box in Fig. 3) and four accessions lacked the deletion. Thirty accessions of *Ae. umbellulata* had no deletion, while three had the deletion. This showed that the deletion cannot distinguish the *Ae. caudata* chloroplast from the *Ae. umbellulata* chloroplast.

PCR data showed that some U chloroplast genomes shared the deletion with the majority of C chloroplast genomes. To determine additional polymorphisms between *Ae. caudata* and *Ae. umbellulata* chloroplast genomes, we sequenced U6/R6 alleles from ten accessions of *Ae. umbellulata* and four accessions of *Ae. caudata* lacking the deletion. All ten accessions of *Ae. umbellulata* had nucleotide T at position 58,329 (bold double-underlined nucleotide in Fig. 3) based on the complete sequence of wheat cpDNA (Ogihara and Tsunewaki 2000). All four accessions of *Ae. caudata* had nucleotide G at the same position. This nucleotide position was presumably genome-specific with T being specific for *Ae. umbellulata* and G specific for *Ae. caudata*.

The observation that *Ae. triuncialis* had two chloroplast types, i.e. with and without the deletion, indicates two origins (Murai and Tsunewaki 1986). Of 14 accessions of *Ae. triuncialis* lacking the deletion, seven of them had nucleotide G, indicating that they had the diploid C genome as maternal parent (one origin). Seven accessions had nucleotide T at the same position, presumably derived from the diploid U genome maternal parent (another origin). At least one additional origin was assumed for the accessions with the deletion. Thus, chloroplast sequence data suggests that at least three origins occurred in *Ae. triuncialis* formation, although one of the events may be due to introgression between *Ae. triuncialis* and a diploid progenitor.

We also sequenced the entire U6/R6 locus from 5 accessions of *Ae. caudata*, 12 accessions of *Ae. umbellulata* and 10 of *Ae. triuncialis* to determine if additional polymorphisms linked to the G/T transversion at position 58,329 might provide additional evidence for two chloroplast types lacking the deletion. A total of 651 bases of DNA sequence were obtained for all alleles. Six parsimony sites, 20 variable sites and two indels were revealed. Two alleles were observed in *Ae. triuncialis* accessions. The first allele contained nucleotide T at position 58,329 (U genome-specific, see above) together with an A at both positions 58,276 and 58,523 (bold single-underlined nucleotides in Fig. 3) based on the complete sequence of wheat cpDNA (Ogihara and Tsunewaki 2000). The same allele was also found in *Ae. umbellulata*. The second allele had nucleotide G at position 58,329 (C genome-specific, see above) along with G and T at positions 58,276 and 58,523, respectively. This

Table 3 Distribution of G43 and U6/R6 alleles among *Ae. triuncialis* accessions. The χ^2 test statistic was calculated to test independence between the G43 and U6/R6 alleles. $\chi^2 = 6.859$; 3 *df*; at $P = 0.0765$

U6/R6	G43		Total
	A allele	B allele	
No deletion	47 [38.3937][a]	55 [63.6189]	102 (0.5484)[b]
Deletion	23 [31.6167]	61 [52.3893]	84 (0.4516)
Total	70 (0.3764)	116 (0.6237)	186

[a] The number in square brackets is the expected value
[b] The number in parenthesis is observed phenotype frequency

allele was also seen in *Ae. caudata*. These two additional nucleotide differences supported our hypothesis that tetraploid *Ae. triuncialis* inherited two distinct chloroplast types lacking the deletion. One allele in *Ae. triuncialis* was inherited from *Ae. caudata* and another allele was inherited from *Ae. umbellulata*. Another observation based on this sequence was a pair of direct repeats flanking the deletion region (bold italicized nucleotides in Fig. 3) that might be responsible for the deletion mechanism (Ogihara et al. 1992).

Nuclear and chloroplast polymorphisms indicated linkage disequilibrium by nuclear and chloroplast correspondence though it was not strongly supported (Table 3; $\chi^2 = 6.859$; 3 *df*; $P = 0.0765$). The U6/R6 chloroplast type with the deletion was more often associated with the G43 B allele (61 accessions) than would be expected (52.39) if these alleles were independent. A plausible explanation is that the U6/R6 (with deletion) chloroplast and the G43 B allele were introduced from a single U parent. Although hybridization has occurred among accessions of independent origin, it has not been sufficient to cause linkage equilibrium among the chloroplast and nuclear sequences.

In conclusion, our results suggest that at least three polyploidization events occurred to form *Ae. triuncialis*. Two alleles are shared between *Ae. umbellulata* and *Ae. triuncialis* based on nuclear DNA sequence at the G43 locus; while two chloroplast types, with and without a 300-bp deletion, are found in both the diploid progenitors and polyploid. Additionally, based on three nucleotide polymorphisms, it appears that the chloroplast type lacking the deletion may have two origins.

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