Fluorescence in situ hybridization karyotyping reveals the presence of two distinct genomes in the taxon *Aegilops tauschii*

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Abstract

**Background:** *Aegilops tauschii* is the donor of the bread wheat D genome. Based on spike morphology, the taxon has conventionally been subdivided into ssp. *tauschii* and ssp. *strangulata*. The present study was intended to address the poor match between this whole plant morphology-based subdivision and genetic relationships inferred from genotyping by fluorescence in situ hybridization karyotyping a set of 31 *Ae. tauschii* accessions.

**Results:** The distribution of sites hybridizing to the two probes oligo-pTa-535 and (CTT)₁₀ split the *Ae. tauschii* accessions into two clades, designated Dt and Ds, which corresponded perfectly with a previously assembled phylogeny based on marker genotype. The Dt cluster was populated exclusively by ssp. *tauschii* accessions, while the Ds cluster harbored both ssp. *strangulata* and morphologically intermediate accessions. As a result, it is proposed that *Ae. tauschii* ssp. *tauschii* is restricted to carriers of the D t karyotype: their spikelets are regularly spaced along the rachis, at least in the central portion of their spike. Accessions classified as *Ae. tauschii* ssp. *strangulata* carry the D s karyotype; their spikelets are irregularly spaced. Based on this criterion, forms formerly classified as ssp. *tauschii* var. *meyeri* have been re-designated ssp. *strangulata* var. *meyeri*.

**Conclusions:** According to the reworking of the taxon, the bread wheat D genome was most probably donated by ssp. *strangulata* var. *meyeri*. Chromosomal differentiation reveals intra-species taxon of *Ae. tauschii*. *Ae. tauschii* ssp. *tauschii* has more distant relationship with breed wheat than ssp. *strangulata* and can be used for breeding improving effectively.

**Keywords:** Chromosome differentiation, D genome, Repeat sequences, Spike morphology, Subspecies, Wheat evolution
The established subspecies structure is not well matched with genetic relationships derived from genotypic characterization. Two distinct phylogenetic lineages, designated L1 and L2, have been recognized [13–15]. The former coincides with ssp. 

The spike morphology of the spike. Individuals classified as ssp. 

FISH karyotyping
Caryopses were imbibed for 24 h at 4 °C, then germinated under a 16 h photoperiod (light/dark temperature 22/16 °C). Root tips of length 1-2 cm were excised and exposed for 2 h to 1.0 MPa NO gas, fixed in glacial acetic acid for at least 5 min, and finally stored in 70% v/v ethanol before preparing slides. The slides were prepared as previously described by Komuro et al. [30] and then denaturated as described by Hao et al. [34]. A 10 μL aliquot of hybridization mixture was applied to each slide, which was allowed to incubate at 37 °C for at least one hour. Finally, a drop of DAPI-containing Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) was added and the preparation was covered with a coverslip. Hybridization signals were observed using an Olympus BX-51 epifluorescence microscope and the images were taken using a Photometric SenSys Olympus DP70 CCD camera (Olympus, Tokyo). Raw images were processed using Photoshop V7.0 (Adobe Systems Incorporated, San Jose, CA). Once the slide had been scored, it was readied for a subsequent stripping and rehybridization following Komuro et al. [30]. The probes included in the hybridization solution were 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (Tamra) labeled oligonucleotides (CTT)_10 (AAC)_5 and (ACG)_5 [28, 35, 36], oligo-pSci19.2, oligo-pTa-535, oligo-pTa71 [31] and oligo-pTa-713 [37], all synthesized by TSINGKE Biological Technology Company (Chengdu, Sichuan, China).

Results
The spike morphology of Ae. tauschii
Based on the appearance of the spike, the 31 Ae. tauschii accessions were classified into three morphological types (Table 1; Fig. 1) as follows:

Type S (ten accessions): these accessions formed markedly moniliform spikes with quadrate spikelets (similar with respect to width and length). Their average SI values ranged from 1.66-1.85 in 2015 in Wenjiang. Individual spikelets of these accessions ranged from 1.52-2.13. The members of this group all belong to ssp. strangulata.

Type T (nine accessions): these accessions formed elongated cylindrical spikes. Their low SI (1.04-1.27) reflected the similar width of their spikelet glume and rachis segment. Individual spikelets of these accessions ranged from 1.00-1.36. The members of this group all belong to ssp. tauschii.

Type I (12 accessions): these accessions were classified as intermediate types, with an SI ranging from 1.40-1.74. Individual spikelets of these accessions ranged from 1.33-2.09. Some (PI603238, PI574465, PI431602, PI603249 and AL8/78) had previously been assigned to ssp. strangulata on the basis of their mildly moniliform spike, but their spikelets were too elongated to be
Table 1  *Aegilops tauschii* accessions

| Code   | Accession* | Origin      | Collector’s taxon | Spike type (SI ± SD)α | FISH group | Our taxon | Sublineageβ |
|--------|-------------|-------------|-------------------|------------------------|------------|----------|-------------|
| 1      | AS2388     | Iran        | ssp. *strangulata* | S (1.72 ± 0.10)ABC   | D4         | ssp. *strangulata* | L2E         |
| 2      | PI603227   | Iran        | ssp. *strangulata* | S (1.59 ± 0.07)F     | D4         | ssp. *strangulata* | L2E         |
| 3      | Clae13     | Iran        | ssp. *strangulata* | S (1.69 ± 0.09)CDE   | D4         | ssp. *strangulata* | L2E         |
| 4      | Clae16     | Iran        | ssp. *strangulata* | S (1.73 ± 0.11)ABC   | D4         | ssp. *strangulata* | L2E         |
| 5      | Clae18     | Iran        | ssp. *strangulata* | S (1.74 ± 0.10)ABC   | D4         | ssp. *strangulata* | L2E         |
| 6      | AS2386     | Iran        | ssp. *strangulata* | S (1.85 ± 0.14)F     | D4         | ssp. *strangulata* | L2E         |
| 7      | AS2403     | unknown     | ssp. *strangulata* | S (1.79 ± 0.08)AB    | D4         | ssp. *strangulata* | L2E         |
| 8      | AS2405     | Iran        | ssp. *strangulata* | S (1.77 ± 0.04)ABC   | D4         | ssp. *strangulata* | L2E         |
| 9      | AS2402     | Israel      | ssp. *strangulata* | S (1.66 ± 0.07)CDE   | D4         | ssp. *strangulata* | L2E         |
| 10     | AS66       | Transcaucasia| ssp. *strangulata* | S (1.76 ± 0.09)ABC   | D4         | ssp. *strangulata* | L2E         |
| 11     | PI603238   | Azerbaijan  | ssp. *strangulata* | I (1.43 ± 0.04)F     | D4         | ssp. *strangulata* | L2 W        |
| 12     | PI574465   | Azerbaijan  | ssp. *strangulata* | I (1.46 ± 0.09)F     | D4         | ssp. *strangulata* | L2 W        |
| 13     | PI431602   | Turkmennistan| ssp. *strangulata* | I (1.41 ± 0.07)FG    | D4         | ssp. *strangulata* | L2 W        |
| 14     | PI603249   | Iran        | ssp. *strangulata* | I (1.60 ± 0.05)EF    | D4         | ssp. *strangulata* | L2 W        |
| 15     | ALB78      | Armenia     | ssp. *strangulata* | I (1.41 ± 0.05)FG    | D4         | ssp. *strangulata* | L2 W        |
| 16     | PI603239   | Azerbaijan  | ssp. *tauschii*    | I (1.67 ± 0.12)CDE   | D4         | ssp. *tauschii*   | L2 W        |
| 17     | PI603233   | Azerbaijan  | ssp. *tauschii*    | I (1.41 ± 0.07)F     | D4         | ssp. *tauschii*   | L2 W        |
| 18     | PI276985   | Iran        | ssp. *tauschii var. meyeri* | I (1.74 ± 0.20)ABC | D4         | ssp. *tauschii var. meyeri* | L2E         |
| 19     | Clae26     | Iran        | ssp. *tauschii var. typica* | I (1.46 ± 0.05)F   | D4         | ssp. *tauschii*   | L2E         |
| 20     | AS63       | unknown     | ssp. *tauschii var. meyeri* | I (1.49 ± 0.07)FG    | D4         | ssp. *tauschii var. meyeri* | L2E         |
| 21     | Clae23     | Iran        | ssp. *tauschii var. meyeri* | I (1.59 ± 0.04)FG    | D4         | ssp. *tauschii var. meyeri* | L2E         |
| 22     | Clae21     | Iran        | ssp. *tauschii var. typica* | I (1.70 ± 0.07)CDE  | D4         | ssp. *tauschii*   | L2E         |
| 23     | PI210987   | Afghanistan| ssp. *tauschii*    | T (1.18 ± 0.03)H     | D4         | ssp. *tauschii*   | L1E         |
| 24     | PI574467   | Russian Federation| ssp. *tauschii* | T (1.04 ± 0.04)F | D4         | ssp. *tauschii*   | L1 W        |
| 25     | AS79       | China       | ssp. *tauschii*    | T (1.21 ± 0.03)F     | D4         | ssp. *tauschii*   | L1E         |
| 26     | AS77       | China       | ssp. *tauschii*    | T (1.18 ± 0.03)F     | D4         | ssp. *tauschii*   | –           |
| 27     | AS2410     | China       | ssp. *tauschii*    | T (1.19 ± 0.04)F     | D4         | ssp. *tauschii*   | –           |
| 28     | AS60       | Iran        | ssp. *tauschii*    | T (1.22 ± 0.05)F     | D4         | ssp. *tauschii*   | L1E         |
| 29     | AS67       | Iran        | ssp. *tauschii*    | T (1.27 ± 0.04)F     | D4         | ssp. *tauschii*   | L1E         |
| 30     | AS68       | Iran        | ssp. *tauschii*    | T (1.25 ± 0.09)F     | D4         | ssp. *tauschii*   | L1E         |
| 31     | Clae1      | Pakistan    | ssp. *tauschii*    | T (1.17 ± 0.04)F     | D4         | ssp. *tauschii*   | L1E         |

* Accessions marked “AS” were obtained from the Triticaceae Research Institute, Sichuan Agricultural University, China; those marked “PI” or “Clae” were obtained from USDA-ARS. α: Types S (ssp. *strangulata*), T (ssp. *tauschii*) and I (intermediate forms). The SI was given by the ratio spikelet glume width/rachis segment width. Capital letters after the average SI value and standard deviations (SD) denote the results of LSD comparison (P < 0.01). β: Genetic lineage data according to marker genotype taken from Wang et al. (2013). L1E (L1 East) and L1 W (L1 West) represent the T type gene pool, and L2E (L2 East) and L2 W (L2 West) the S type gene pool.

unequivocally assigned to this taxon. Accessions PI276985, AS63 and Clae23 (assigned previously to ssp. *tauschii var. meyeri*) formed elongated spikelets, but their spikes were not sufficiently cylindrical and their rachis segments too narrow to fit this taxon.

**FISH markers specific for two typical ssp.**

Seven probes (Fig. 2) were preliminarily hybridized in situ to mitotic chromosome spreads of nine accessions, including the two from S type (AS2388 and AS2402), two from I type (AS63 and PI431602), and five from T type (AS68, AS77, AS79, Clae1 and PI210987). However, the probes oligo-pTa-713, (AAC)_5 and (ACG)_5 failed to reveal any subspecies-specific difference for the analyzed materials. As a result, these three probes were not used any further. Although oligo-pSc119.2 also failed to detect subspecies-specific difference, it was helpful to identify chromosomes 2D, 3D, and 4D. Oligo-pSc119.2 and the remaining three probes were tested on mitotic chromosome spreads of the full set of *Ae. tauschii* accessions as well as on CS.
**Oligo-pTa71**

This sequence hybridized to the nucleolar organizing region on chromosome arm 5DS in all of the *Ae. tauschii* accessions. The hybridization signal strength was higher in the Type T than in the Type S accessions (Fig. 3). However, the probe did not hybridize strongly with the CS 5D chromosome.

**Oligo-pSc119.2**

This probe hybridized to sites close to the telomere of chromosome arms 1DS, 2DS, 3DS and 4DS (Fig. 4). The pattern of hybridization did not discriminate between the Types S, T and I, but was useful as a means of identifying individual chromosomes.

**(CTT)_{10}**

This probe hybridized to sites on chromosome arms 1DS, 2DS, 3DS and 4DS, and the patterns were informative with respect to the three Types (Fig. 3). The four chromosome arms harbored a hybridization site in all Type S accessions, but these sites were absent from all Type T accession karyotypes. A site in the sub-telomeric region of chromosome arm 1DL was present in both the Type T accessions AS67 and AS68 (from Iran) and Clae1 (Pakistan). A further site in the sub-telomeric region of chromosome arm 2DS was restricted to just three Type T accessions (AS77, AS79, AS2410) all originating from central China, the most easterly reach of the species.

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**Fig. 1** Morphological variation within *Ae. tauschii*. (a) Spikes, (b, c) spikelets. Types S (ssp. *strangulata*), T (ssp. *tauschii*) and I (intermediate forms) are distinguished by the width of the rachis segment (blue bar in #23) and the glume (red bar in #23). In (c) the measurement points for spikelet glume width (G) and rachis segment width (R) used to derive Si are indicated. The numbers 1-31 in (b) relate to accessions 1-31 in Table 1.

**Fig. 2** FISH karyotype, based on seven probes, of (a) the Type S accession AS2402, (b) the Type T accession AS79.
Oligo-pTa-535

The oligo-pTa-535 probe hybridized to many chromosomal sites, allowing it to be used as a means discriminating each of the seven of *Ae. tauschii* chromosomes (Fig. 4). Type-specific sites were observed on chromosome arms 2DS, 3DL, 4DS, 5DL and 6DL. Both the middle region of chromosome arm 2DS and the sub-telomeric region of chromosome arm 4DS included hybridization sites which were unique to Type T accessions, while one site in the sub-telomeric region of chromosome arm 3DL and another in the centromeric region of 5DL were absent from all Type T accessions. The 5DL arm harbored several hybridization sites, but their distribution along the arm differed between Type S and Type T accessions. A similar Type-specific distribution of sites was detected in the region stretching from...
about two thirds of the way along chromosome arm 6DL to its telomere.

The FISH karyotype of type S and type I accessions
The karyotypes of Type S and Type I accessions were quite similar to one another. The four (CTT)$_{10}$ sites present in all Type S (but in no Type T) accessions were represented in most of the Type I karyotypes; the exceptions were the chromosome arm 1DS site (missing in PI603249), the 3DS site (missing in PI603249 and Clae21) and the 4DS site (missing in Clae21, AS63 and Clae23) (Fig. 3). The absence of the 1DS and 3DS sites in PI603249 was accompanied by lengthened chromosome 1D, a foreshortened chromosome 3D and a unique hybridization site on chromosome 3D (Fig. 5a), which might be indicative of a major structural alteration in the genome. In Clae21, both chromosomes 4D and 5D were unusual in length (Fig. 5b). All three Type I accessions lacking the 4DS site formed slender spikelets (Fig. 1), used as a diagnostic for var. meyeri, although Clae21 was classified as var. typica by its collector (Table 1). The sub-telomeric region of chromosome arm 6DS of the three Type I accessions PI431602, Clae26 and AS63, along with that of the Type S accession PI603227, harbored a further (CTT)$_{10}$ site (Fig. 3). The distribution of oligo-pTa-535 sites confirmed the closeness of the relationship between Types S and I. In both the middle region of chromosome arm 2DS and the sub-telomeric region of chromosome arm 4DS, sites present in the Type T karyotype were missing in both Type S and I, while the Type S-specific sites close to the chromosome arm 3DL telomere were also present in Type I (Fig. 4).

The weakly hybridizing sites lying on chromosome arm 5DL close to the centromere were present in both Type S and I accessions. Similarly, the chromosome arm 6DL region was more similar between Types S and I than between Types S and T. Overall, the karyotypic analysis provided evidence that Type I accessions are genetically closer to Type S than to Type T.

The FISH karyotype of Ae. tauschii in relation to that of CS
Probe (CTT)$_{10}$ hybridized to sites on chromosome arms 1DS and 2DS in the Type S and I accessions and also to the same location in CS (Fig. 3). However, neither the chromosome arm 3DS nor 4DS sites were present in CS, which was also a feature of the Type I accession Clae21, while AS63 and Clae23 lacked the 4DS site, but harbored the 3DS one. The (CTT)$_{10}$ sub-telomeric sites on 7DS were only present in the Type S accession Clae16, Type I accession PI276985, and CS (Fig. 3). With respect to oligo-pTa-535, the karyotype of CS (as also that of the Type S and Type I accessions) lacked the sites at middle region on 2DS and the sub-telomeric region of 4DS, and shared a similar distribution of sites with the Types S and I accessions on chromosome arms 3DL, 5DL and 6DL and around the chromosome 5D centromere (Fig. 4).

Discussion
Although it was thought at one time that repetitive DNA had no function, it is now believed that it does play some role in chromosome organization and stabilization, mitosis and meiosis, and even gene regulation [38]. Both the amount and distribution of such

![Fig. 5](image-url) Chromosome variants with oligo-pSc119.2 (red) and oligo-pTa-535 (green). a #14 (PI603249) harbors a longer chromosome 1D and a shorter chromosome 3D; b #22 (Clae 21) harbors a shorter chromosome 5D, and a version of chromosome 4D showing an unusual distribution of hybridization sites.
sequences act to drive evolution and speciation [39], so that their diversification has been used to assess genetic relatedness at both the species and the genome level [40, 41].

Genome heterogeneity within *Ae. tauschii*

FISH karyotyping based on two sequences (oligo-pTa-535 and (CTT)$_{10}$) was able to reveal intraspecific differentiation within *Ae. tauschii*. Two distinct forms of the D genome were recognized from the karyotypic analysis of the 31 accessions (Figs. 3 and 4). One, present in nine accessions categorized as ssp. *tauschii*, is designated here D$^t$, and the other, present in the ssp. *strangulata* and L2 are recognizably different from one another, and L1/Dt corresponds to ssp. *tauschii* fits the intermediate type (Type I). The conclusion is that accessions exhibit a spike morphology which better represents the D$^t$ and D$^s$ evolved either from independent hybridization events or via segregation from a common progenitor hybrid.

An analysis based on single nucleotide polymorphisms has suggested that the donor of the bread wheat D genome was a member of the L2 lineage [15], to which the nine of the ten Type S accessions along belong (Table 1). The other five L2 members (PI276985, CIae26, AS63, Clae21 and Clae23) are all Type I, now re-designated as ssp. *strangulata* var. *meyeri*. One of these (Clae23) maps closest to the bread wheat D genome [15]. Given that the distribution of (CTT)$_{10}$ sites across the CS D genome more closely resembled that seen in Clae21, Clae23 and AS63 (Fig. 3), the conclusion is that the progenitor of the bread wheat D genome was likely a member of ssp. *strangulata* var. *meyeri*.

Wheat D genome genetic improvement

Further genetic improvement is needed to match wheat production to an increasing global demand. Accessing novel genetic variation from wheat’s D genome wild relatives has proved to be a highly successful strategy in recent years [44]. Probably very few *Ae. tauschii* individuals were involved in the original natural hybrids which were the progenitors of modern bread wheat, creating a major evolutionary bottleneck. Thus, of over 7000 D genome single nucleotide polymorphism sites, about 99% have been contributed by lineage L2 [15]. The implication is that a future priority should be to drive introgression from L1, using accessions such as AS60. The Chinese cultivar Shumai 969 released in 2013 was bred by using a synthetic hexaploid formed by crossing AS60 with *Triticum turgidum* [45]. It has rapidly become a leading cultivar in Sichuan Province, China, and demonstrates the potential of carriers of the D$^t$ genome for wheat improvement.

Conclusions

According to the reworking of the taxon, the bread wheat D genome was most probably donated by ssp. *strangulata* var. *meyeri*. Chromosomal differentiation reveals intraspecies taxon of *Ae. tauschii*. *Ae. tauschii* ssp. *tauschii* has more distant relationship with breed wheat than ssp. *strangulata* and can be used for breeding improving effectively.
Abbreviations
Ae: Aegilops; CS: Chinese Spring; FISH: Fluorescence in situ hybridization; G: Glume width; R: Rachis segment width; SI: Subspecies index

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files.

Author contributions
D. L., L. Zhao, Y. Z., S. N. and M. H. planned and designed the research. L. Zhao, Y. Y., S. N. and Z. Y. performed experiments. J. W. and L. Zhang analyzed data. D. L., L. Zhao and M. H. wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
Plant materials with AS codes are from the Triticeae Research Institute, Sichuan Agricultural University, China; accesses with codes PI or Ciae were from USDA-ARS, USA. Experimental research on plants complies with the guidelines of the People’s Republic of China.

Consent to publication
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Competing interests
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