Aegilops tauschii Coss. is the D-genome donor to hexaploid bread wheat (Triticum aestivum) and is the most promising wild species as a genetic resource for wheat breeding. To study the population structure and diversity of 81 Ae. tauschii accessions collected from various regions of its geographical distribution, the genomic representation of these lines were used to develop a diversity array technology (DArT) marker array. This Ae. tauschii array and a previously developed DArT wheat array were used to scan the genomes of the 81 accessions. Out of 7500 markers (5500 wheat and 2000 Ae. tauschii), 4449 were polymorphic (3776 wheat and 673 Ae. tauschii). Phylogenetic and population structure studies revealed that the accessions could be divided into three groups. The two Ae. tauschii subspecies could also be separately clustered, suggesting that the current taxonomy might be valid. DArT markers are effective to detect very small polymorphisms. The information obtained about Ae. tauschii in the current study could be useful for wheat breeding. In addition, the new DArT array from this Ae. tauschii population is expected to be an effective tool for hexaploid wheat studies.

Key Words: Aegilops tauschii, Triticum aestivum, synthetic wheat, DArT markers, drought tolerance.
The diversity of *Ae. tauschii* has been studied using molecular tools such as chloroplast DNA variation (Matsuoka et al. 2008, 2009, Takumi et al. 2009), AFLP (Mizuno et al. 2010), SSR (Naghavi and Mardi 2010), isozymes (Dudnikov and Kawahara 2006) and random amplified polymorphic DNA (RAPD) markers (Okuno and Kawahara 2006). Here, we used diversity array technology (DArT) markers to study the population structure and diversity of *Ae. tauschii*. DArT is a sequence-independent system, based on microarray hybridization that can be used to carry out a whole-genome scan. The method is based on the use of “genomic representations”, which are DNA samples produced by using a specific combination of restriction enzymes and PCR primers. The output result is (0, 1); that is, it indicates the presence or absence of each DNA fragment contained in a genomic representation within the genome of the material being tested. DArT markers are biallelic dominant markers that provide a cost-effective, time-saving method of genome-wide genotyping (Jaccoud et al. 2001, Kilian et al. 2005). These markers have been successfully used for genotyping, diversity studies, and genetic mapping in many crop species (Jing et al. 2009).

The molecular information provided by the present DArT analyses will be useful for breeding programs.

**Materials and Methods**

**Plant materials and DNA isolation**

Eighty-one accessions of *Ae. tauschii* collected from various regions of its geographical distribution were used in this study. Thirteen of the accessions were ssp. *strangulata*, 62 were ssp. *tauschii*, and six were of unknown subspecies (Table 1). The AE accessions were collected by the Institut für Pflanzengetenkt und Kulturpflanzenforschung (IPK), Germany; the AT accessions by the Faculty of Agriculture, Okayama University, Japan; the CGN accessions by the Institut voor Plantenveredeling, Landbouwhogeschool Wageningen, the Netherlands; the IG accessions by the International Center for Agricultural Research in the Dry Areas (ICARDA), Syria; the KU accessions by the Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan; and the PI accessions by the United States Department of Agriculture (USDA). Fifty-five of these *Ae. tauschii* accessions have been used for producing synthetic wheat. These *Ae. tauschii* were kindly provided by Prof. Y. Matsuoka, Fukui Prefectural University, Japan. The accession 28H51 has been used by ICARDA for producing synthetic wheat derivatives. Accession Aus18913 has been used for mapping of chromosome arm 1DS of *Ae. tauschii* (Spielmeyer et al. 2000) and to construct an *Ae. tauschii* BAC library (Moulet et al. 1999). Total genomic DNA was extracted from 2- to 3-week-old leaves of the 81 accessions by using the CTAB method (Murray and Thompson 1980).

**Preparation of DArT markers and genotyping**

A total of 81 genotypes DNA were sent to Diversity Arrays Technology Pty. Ltd., Yarralumla, Australia, for array development and genotyping as described by Wenzl et al. (2004) and Akbari et al. (2006). The *Ae.tauschii* DArT markers are referred to as (aefP-). Each of the original 81 accessions was genotyped with two arrays: Wheat DArT Array Version 3.0 and the *Ae. tauschii* array developed in this study. The genomic DNA of each accession was labeled and then hybridized to the DArT arrays. The presence or absence

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### Table 1. *Ae. tauschii* accessions used in this study

| Country (number of accessions) | Accession* |
|-------------------------------|------------|
| Afghanistan (n = 6)            | KU2039, PI476874, KU2063, KU2035, KU2022, KU2636^a |
| Armenia (n = 7)                | IG127015, KU2816, KU2810, KU2824, CGN10734, KU2809^a, IG126991 |
| Azerbaijan (n = 2)             | IG47203, KU2806 |
| China (n = 6)                  | AT55, AT76, AT80, AT47, PI499262, PI508262 |
| Dagestan (n = 2)               | KU201, IG120866 |
| Georgia (n = 5)                | AE454, KU2826, KU2828, KU2829A, IG48042 |
| India (Jammu and Kashmir) (n = 1) | IG48042 |
| Iran (n = 37)                  | KU20-10^M, KU2069, KU2074^a, KU2075^a, KU2076^a, KU2078^a, KU2079^a, KU20-8, KU2080^a, KU2088^a, KU20-9^a, KU2090^a, KU2091^a, KU2092^a, KU2093, KU2096, KU2097, KU2098, KU2100^M, KU2103, KU2104, KU2105, KU2106, KU2109^M, KU2111, KU2124, KU2126, KU2144, KU2155, KU2156, KU2158^M, KU2159, KU2083, KU2101, KU2102, KU2108^M, IG49095 |
| Kazakhstan (n = 1)             | AE1090 |
| Kyrgyzstan (n = 1)             | IG131606 |
| Pakistan (n = 4)               | IG46663, CGN10768, CGN10770, CGN10767 |
| Syria (n = 2)                  | IG46623, 28H51 |
| Tajikistan (n = 1)             | IG48554 |
| Turkey (n = 3)                 | KU2132, KU2136, PI486277 |
| Turkmenistan (n = 2)           | IG126387, IG126489 |
| Unknown (n = 1)                | Aus18913^M |

*Accessions without superscripts are ssp. *strangulata*; accessions with superscripts are ssp. *tauschii*^a^, ssp. *tauschii var. anathera*^M^, ssp. *tauschii var. meyeri*^a^, ssp. *tauschii var. strangulata*. 
of each marker was determined on the basis of the signals from the labeling and image analysis. The DArT marker data were provided to in term of 1, 0 (present, absent) fashion, as described by Akbari et al. (2006).

Analysis of DArT data

To determine the population structure of *Ae. tauschii*, we applied the Bayesian method by using the model-based program Structure version 2.3.3 (Falush et al. 2003, Pritchard et al. 2000) with (0, 1) data matrices. We used a burn-in length (number of cycle runs by the simulation before collecting data) of $10^4$ cycles to minimize the effect of starting configuration, and a simulation run length (after the burn-in) of $10^6$ cycles, and applied the admixture model option in the Structure program. We chose cluster values (K) ranging from 2 to 9; four independent runs for each value gave consistent results.

Genetic similarities between accessions were measured by DICE similarity coefficient based on the proportion of shared alleles. The phylogenetic tree was constructed by clustering accessions based on similarity matrix using the unweighted pair group method (UPGMA) with arithmetic average algorithm in the SAHN module. Bootstrap analysis was performed using 1,000 permutations in Winboot (Yap and Nelson 1996). Bootstrap values over 50 are considered significant and indicated on the phylogenetic tree.

To calculate the genetic similarities and genetic distances between the pairs of accessions, the (0, 1) data matrices obtained for 4449 polymorphic DArT markers (details presented in the Results section) and 81 accessions were analyzed with the following formulae:

\[
S_{ij} = \frac{(2 \times N_{ij})}{(N_i + N_j)}
\]

\[
D_{ij} = 1 - \frac{[(2 \times N_{ij})]}{(N_i + N_j)}
\]

Where, $S_{ij}$ represents the similarity between the i-th and j-th accessions, $N_{ij}$ represents the number of common bands present in both the $N_i$ and $N_j$ accessions (i.e., the number of markers where 1’s are present for both the $N_i$ and $N_j$ accessions), $N_i$ is number of bands in the i-th accession (number of markers with 1’s in accession $N_i$), and $N_j$ is number of bands in the j-th accession (number of markers with 1’s in accession $N_j$).

By using the program Excel (Microsoft Corporation), we calculated genetic similarity for all 3240 possible pairs ($81 \times (81-1)/2$) of the 81 accessions using the (0, 1) data matrix consisting of 4449 rows (4449 DArT markers) and 81 columns (81 accessions). An $81 \times 81$ genetic similarities matrix was created in which the values of 1 on the main diagonal (representing the similarity of each accession to itself) were not considered. The values in the genetic similarities matrix were used to calculate average similarity within ssp. *strangulata* and within the three varieties of ssp. *tauschii*. The average similarities between ssp. *strangulata* and each of the varieties of ssp. *tauschii* were also calculated.

### Results

**Polymorphism of DArT markers in *Ae. tauschii* accessions**

A total of 7500 DArT markers were tested. Of these, 5500 markers from wheat DArT Array Version 3.0 (wheat markers) were developed from hexaploid bread wheat and the other 2000 (*Ae. tauschii* markers) were developed from the 81 *Ae. tauschii* accessions by the DArT company (Table 2). Of these markers, 3776 of the wheat markers (68.6%) and 673 of the *Ae. tauschii* markers (33.7%) showed polymorphism among the 81 accessions of *Ae. tauschii*. The polymorphism information content (PIC) of the DArT markers ranged from 0.024 to 0.500 per marker, with an average of 0.259 (Table 2). Thirty-two markers had a very low PIC (0.024), which means that these markers showed little polymorphism. About 96% of the markers had a call rate of more than 90% and 48% of the markers had a call rate of 100%; 95% of the wheat markers had a call rate of 90% or more while almost all of the *Ae. tauschii* markers had a call rate of more than 87%. To verify the reproducibility of the genotypic variation, two of the accessions were analyzed in duplicate (i.e., two wells per accession). The results of both pairs were identical, except for a few missing data points.

**Phylogenetic tree based on DArT marker genotypes**

Phylogenetic trees were constructed using the 3776 polymorphic wheat markers, the 673 polymorphic *Ae. tauschii* markers and the combined total of 4449 markers showing polymorphism. The structures of these three trees were very similar, therefore, the tree made by using all 4449 markers is shown in Fig. 1.

**The *Ae. tauschii* population could be clearly divided into three groups designated A, B and C. Group A contained accessions from China, central Asia (Kazakhstan, Kirghizistan, Tajikistan and Turkmenistan), Afghanistan, Pakistan, India and western Asia (Georgia, Armenia, Syria) and only three***

### Table 2. Quality parameters of the two different types of DArT markers used to analyze 81 *Ae. tauschii* accessions

| Parameter                          | Wheat Array 3.0 | *Ae. tauschii* | Overall |
|------------------------------------|----------------|---------------|---------|
| Total number of DArT markers       | 5500           | 2000          | 7500    |
| Number of polymorphic markers      | 3776           | 673           | 4449    |
| Polymorphism (%)                   | 68.6           | 33.7          | 59.3    |
| Polymorphism information content   | 0.246          | 0.329         | 0.259   |
| P (%)                              | 81.2           | 82.9          | 81.5    |
| Reproducibility (%)                | 99.9           | 99.8          | 99.9    |
| Call rate (%)                      | 97.8           | 97.6          | 97.8    |

*a* Number of polymorphic markers/total number of DArT markers tested.

*b* Measure of polymorphism to describe the usefulness of a marker.

*c* Reflects how well the two phases of the marker are separated.

*d* On the basis of scoring for replicated samples.

*e* The number of genotypes present and not missing for certain marker.
The diversity analysis of *Aegilops tauschii* by DArT markers

Accessions (KU2144, IG49095 and KU2109) were from Iran. Group B had three accessions, two from Georgia and one from Afghanistan. Accessions in group C were from Iran, with the exception of accession KU2828, IG46623, IG120866 and IG127015, which were from western Asia (Georgia, Syria, Dagestan and Armenia, respectively).

Sixty-six of the accessions in this study belonged to ssp. *tauschii*, of which 58 were var. *typica*, 6 var. *meyeri* and 2 var. *anathera* (Fig. 1). Twelve accessions were classified as ssp. *strangulata* and the remaining 3 accessions were of unknown variety. All of the accessions of ssp. *strangulata* clustered in one clade in Group C. On the other hand, the three varieties of ssp. *tauschii* did not cluster into a particular clade. Accessions classified as var. *anathera* were present only in Group A, while those classified as var. *meyeri* appeared in Group C, with only one (KU2109) in Group A. Using this tree, we could deduce that the 3 accessions without species information belong to ssp. *tauschii*. Accession IG127015 from Armenia was classified into Group C but was separate from the other accessions in this group. Accessions KU2039 (Afghanistan), AE454 (Georgia) and KU2829 (Georgia) were classified into Group B (Fig. 1).

**Structural analysis of the Ae. tauschii population**

The structure of the *Ae. tauschii* population was further studied to assess the degree of relatedness among the accessions and to group genetically similar accessions (Fig. 2). For this purpose, we used the model-based program Structure version 2.3.3 (Falush et al. 2003, Pritchard et al. 2000). Four independent runs yielded consistent results. Values of $K$ (number of clusters) ranging from 2 to 9 were tested. The values log-likelihood for the observed data from $K = 2$ to 9 are: $-77679.6$, $-72394.1$, $-538662.9$, $-1840057.9$, $-111610.2$, $-4742273$, $-958412$ and $-1890751$, respectively. $K = 3$ was selected as having the highest log-likelihood for the observed data, indicating that the current *Ae. tauschii* population can be clearly divided into three groups (Fig. 2).

One of the three groups contained the accessions KU2829A, AE454 and KU2039, which are quite distant from the others. Interestingly, these lines carry useful traits for drought tolerance (Sohail et al. 2011, discussed later in detail). The third group corresponds to Group C in the phylogenetic tree, which includes all of the accessions of ssp. *strangulata* and about half (29) of the accessions of ssp. *tauschii*.

**Genetic relatedness and dissimilarity among subspecies and varieties**

We also analyzed the relatedness and dissimilarity between the two subspecies of *Ae. tauschii* and among the three varieties in ssp. *tauschii*. The average similarity among
those in *ssp. strangulata* was 0.87, while that in *ssp. tauschii* was 0.74. Though the average similarity between accessions of var. *typica* and accessions of *ssp. strangulata* was low, the range was wide (0.61 to 0.99), indicating that some of *ssp. tauschii* var. *typica* had great similarity with some accessions of *ssp. strangulata*. Another interesting observation that var. *typica* had very high maximum similarity (0.99) with var. *meyeri* and *ssp. strangulata*. Some of the * Ae. tauschii* accessions had very high genetic similarities (0.99): AT47, AT76 and AT80; KU2096 and KU2098; KU2156 and KU2155; KU2088 and KU2090; KU2108 and KU2103; Aus18913 and KU2102; CGN10767 and CGN10768; KU2076 and KU2078; KU2816 and KU2824 and KU2069 and KU2828.

**Discussion**

**Polymorphism of DArT markers from wheat and *Ae. tauschii***

The DArT method for performing whole-genome scans is proving to be useful for the studies of many plant species. It is currently being used for diversity studies, population structure analysis, mapping, marker-assisted selection for multiple phenotypic traits, etc. (Howard et al. 2011). We tested 5500 previously developed wheat markers (Wheat Array 3.0) and 2000 *Ae. tauschii* markers that were produced in this study to examine the diversity of *Ae. tauschii*. Out of the 7500 total markers tested, 4449 showed polymorphism among the 81 accessions in this study (Table 2). The wheat markers showed a higher percentage of polymorphism (68.6%) than the *Ae. tauschii* markers (33.7%). This might be because the wheat markers had previously been screened and selected as good markers for the D genome of common wheat. However, Pestsova et al. (2000) also had a similar result in a study using SSR markers that had not been prescreened and preselected: the SSR markers developed based on *T. aestivum* sequences revealed a higher level of polymorphism in *Ae. tauschii* accessions than the markers developed from *Ae. tauschii* itself.

Previous studies using DArT markers in cultivated crops showed less polymorphism than we found in the current study; for example, the polymorphism rate in hop (*Humulus lupulus* L.) was 11.9% (Howard et al. 2011); sugarcane (*Saccharum officinarum*), 7.0% (Heller-Uszynska et al. 2010); wheat (*T. aestivum*), 9.4% (Akbari et al. 2006); cassava (*Manihot esculenta*), 14.6% (Xia et al. 2005) and barley (*Hordeum vulgare*), 10.4% (Wenzl et al. 2004). Badea et al. (2011) reported the polymorphism rate of triticale for DArT markers of hexaploid wheat, triticale, and rye as 8.6%, 23.4% and 23.8%, respectively. Compared to these studies, we found higher polymorphism rate for *Ae. tauschii*. This might be because *Ae. tauschii* is a wild species and because the collection used here is representative of the wide diversity of *Ae. tauschii*. DArT markers of related species can be used for crops and vice versa; for example, Jing et al. (2009) reported that *T. monococcum* DArT markers can be effectively used for hexaploid and tetraploid wheat as well as for diploid *Triticum* species.

**Classification of *Ae. tauschii* accessions on the basis of country of origin**

The phylogenetic tree of *Ae. tauschii* made in this study contained three largely differentiated groups, A, B and C. Most accessions in Group A were from regions other than Iran. The region encompassed by Group A starts at the Afghanistan–Turkmenistan border, extends through southern Uzbekistan, Tajikistan and Kirghizstan and reaches all the way to southern Kazakhstan. Some patches of this group are distributed along the Pakistan–Afghanistan border, in India (Jammu and Kashmir), and in western and central China. Group B only had three accessions (AE454 and KU2829A from Georgia and KU2039 from Afghanistan)
these accessions also formed an independent group in the structure analysis. Two of these lines (AE454 and KU2829A) were reported to be in an unclear genealogical position by Matsuoka et al. (2008, 2009) and Mizuno et al. (2010). Most accessions in Group C were from Iran, mainly around the lower western and southern sides of the Caspian Sea and along the Iran–Turkmenistan border. Mizuno et al. (2010) has also reported a similar grouping on the basis of AFLP results. It is noteworthy that the accessions in Group C were collected from areas with a Mediterranean climate whereas the accessions in Group A were collected from areas with an arid or semiarid steppe climate.

Most of the accessions originating from the same country were clustered together in the phylogenetic tree (Fig. 1). Among the six accessions from China, two (PI499262 and PI508262) were clustered together with those from Afghanistan and Kirghizistan and four (AT55, AT80, AT76 and AT47) were clustered together with those from Afghanistan and Turkmenistan. The first two were collected from Xinjiang, which borders with Afghanistan and Kirghizistan, and the other four were from Shaanxi province. The accessions from Xinjiang must have been found in the natural distribution area of this species. However, the accessions from Shaanxi might be weedy types that were carried from Turkmenistan by human activity at some point in history. Likewise, some accessions showed high genetic similarity despite being collected from distant sites. For example, KU2069 from Iran and KU2828 from Georgia were very similar (0.99). This suggests the occurrence of migration, as Ae. tauschii has a weedy growth habit and can occur in a mixture with wheat.

The origin of Aus18913, a key accession for genome sequencing, was known when we began our study. However, our data clearly indicated that it originated from Iran because of its close similarity (0.99) to KU2102, which was collected at 52 km northwest of Ramsar, Iran, on the southwestern coast of the Caspian Sea (http://www.shigen.nig.ac.jp/wheat/komugi/strains/nbrpDetailAction.do?strainId=KU-2102).

Classification of subspecies

Accessions classified as ssp. strangulata clearly clustered together in the phylogenetic tree (Fig. 1), despite the difficulty of classification between ssp. strangulata and ssp. tauschii based on phenotype (Dudnikov and Kawahara 2006, Dvorak et al. 1998, Pestsova et al. 2000), the DArT markers revealed a clear cluster of ssp. strangulata. This indicates that both subspecies are genetically well diverged and that this taxonomy is probably valid. Diversity analysis using thousands of DArT markers is more powerful than analysis with other markers because of the high number and high degree of polymorphism detected by these markers. Two ssp. meyeri accessions, Aus18913 and KU2108, clustered with accessions KU2102 and KU2103 of var. tauschii respectively, and had a similarity of 0.99 with each other. The reason for this might be a large number of traits with intermediate morphology (Dudnikov and Kawahara 2006, Pestsova et al. 2000, van Slageren 1994) caused by hybridization between the two subspecies (Dvorak et al. 1998, Hammer 1980, Hammer and Knupfer 1979).

In ssp. tauschii, the varieties anthera, meyeri and typica could not be well differentiated based on the DArT markers. Mizuno et al. (2010) suggested the reason for this might be that only a small number of genetic loci control the morphological traits used to discriminate between them. Though accessions of var. anthera were included only in Group A, accessions of var. meyeri and var. typica appeared in both groups. On the basis of AFLP markers, Lubbers et al. (1991) reported that ssp. strangulata was more similar to var. meyeri than to var. typica. Here, no accessions of var. meyeri were closely grouped in the phylogenetic tree (Fig. 1).

Use of Ae. tauschii diversity information for wheat breeding

Because Ae. tauschii is the D-genome donor to hexaploid bread wheat, it is regarded as the most promising wild species as a genetic resource for wheat breeding (Feldman 2001, Helbaek 1959, Kihara 1944, Mujeeb-Kazi et al. 1996). To introduce useful genes from this wild species into common wheat, Ae. tauschii (DD) is crossed with durum wheat (AABB) to produce a hexaploid amphiploid (AABBDD) that is called synthetic wheat (has the same genomes as bread wheat). When using this process, we should utilize the large diversity of Ae. tauschii as efficiently as possible. Sohail et al. (2011) measured the morphological and physiological traits related to drought tolerance in many Ae. tauschii accessions and their synthetic hexaploid produced by crosses between Ae. tauschii accessions and a durum wheat, Triticum durum cv. Langdon. The results showed great diversity in drought response at both the diploid and the hexaploid levels. They found that synthetic wheats made by accessions from Georgia and central Asia (corresponding to Group B in this study) showed higher performance than others and that these synthetic wheats and may be useful for wheat breeding. The molecular information provided by the present DArT analyses will elucidate the genetic basis of the morphological and physiological characteristics at both ploidy levels. DArT makers have shown some lines to have very high genetic similarity (0.99); this information is very important for breeders to select suitable and more diverse material for breeding programs, to avoid duplication and save time and labor. This information is also important for gene banks to avoid preserving the same genotypes collected by different researchers and tagged with different accession numbers. For example, some of the accessions having high genetic similarity mentioned in the result section might be duplicates.

In conclusion, DArT markers are capable of detecting even very small polymorphisms, are cost-effective and are efficient for whole-genome scans and population structure. The present study will be useful for wheat breeding and will provide useful information with which to choose a range of accessions that represent a high degree of genetic diversity.
Additionally, the new array developed here, which represents a large and diverse collection of Aegilops tauschii accessions, could be an effective tool for hexaploid wheat studies.

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