Tibet is one of the centers of domestication of cultivated barley

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The Near East Fertile Crescent is well recognized as a primary center of barley origin, diversity, and domestication. A large number of wild barleys have been collected from the Tibetan Plateau, which is characterized by an extreme environment. We used genome-wide diversity array technology markers to analyze the genotypic division between wild barley from the Near East and Tibet. Our results confirmed the existence of Tibetan wild barley and suggested that the split between the wild barleys in the Near East and those in Tibet occurred around 2.76 million years ago (Mya). To test the concept of polyphyletic domestication of barley, we characterized a set of worldwide cultivated barley. Some Chinese hulless and six-rowed barleys showed a close relationship with Tibetan wild barley but showed no common ancestor with other cultivated barley. Our data support the concept of polyphyletic domestication of cultivated barley and indicate that the Tibetan Plateau and its vicinity is one of the centers of domestication of cultivated barley. The current results may be highly significant in exploring the elite germplasm for barley breeding, especially against cold and drought stresses.

adaptation | evolution | harsh environment

Wild barley (*Hordeum spontaneum*) is the progenitor of cultivated barley (*Hordeum vulgare*), which has been one of the founder crops of old world Neolithic food production (1). The Near East Fertile Crescent is one of the earliest sites in terms of world crop domestication, and it is the center of origin and diversity of some valuable wild cereals, e.g., wild wheat and barley, the basis for human civilization (1). The geographic range of wild barley in the Near East was clearly identified, and its relation to barley domestication was proven by genetic and chromosomal studies (1–7). However, increasing evidence supported multiple origins of cultivated barley (8–10). For instance, the fixation of nonbrittle rachis, controlled by two closely linked complementary genes, occurred in at least two centers of barley domestication, East and West (8, 11). Many comprehensive works were done on barley accessions from the Fertile Crescent and Central Asia (9, 12–15). However, most Western researchers had little access to the domestication of barley from the East.

The Tibetan Plateau, generally called "the roof of the world" because of its very high altitude, is characterized by its extreme environment. A large number of wild barleys have been collected from Tibet, and they display wide genetic diversity and close genetic homology to cultivated barley (16–20). However, its contribution to evolution and domestication of barley has long been underestimated.

Genomic diversity provides the basis of evolutionary change by natural selection and domestication (7). Genome-wide marker analysis is currently one of the most powerful tools for determining genetic variation of a crop species and its phylogenetic relationships with wild relatives (1, 21). Diversity array technology (DArT) markers are widely accepted as powerful whole-genome profiling markers for phylogenetic and population structure analysis (13, 22–25). Similar results were obtained in 185 barley accessions using both 1,130 DArT and 1,307 single nucleotide polymorphism (SNP) markers, respectively (13, 14).

In the current study, we analyzed the genetic division between wild barleys from the Near East and Tibet and between wild barley and cultivated barley from the different regions using DArT markers and SNPs with the aim of (*i*) testing the concept that barley domestication is polyphyletic and (*ii*) determining whether Tibet is one of the centers of domestication of cultivated barley.

Results

DArT Marker Polymorphism. Our results showed that 94.5%, 93.7%, and 94.7% of the selected 1,309 DArT markers were polymorphic in cultivated barley, wild barley from the Near East (Wb-NE), and Tibet (Wb-T), respectively (Fig. 1). The polymorphism information content (PIC) for the 1,136 common markers ranged from 0.008 to 0.500, with an average of 0.388 for all examined accessions and genotypes, and 0.379 and 0.353 for the wild and cultivated barleys, respectively, showing wide marker polymorphism and genetic divergence. The mean marker distance was only 1.56 cM (Table 1), indicating a genome-wide coverage of DArT markers and their suitability for evolutionary analysis. Interestingly, although there were 1,136 common markers for all of the barleys used in this study, 37 and 60 polymorphic markers, belonging to the private markers of Wb-NE and Wb-T, respectively, were detected in the cultivated barley (Fig. 1), indicating that the gene pool of the cultivated barley is contributed by both of the wild barleys from the Near East and Tibet.

Genetic Division Between Wild Barleys from the Near East and Tibet. Cluster analysis revealed three ecological types of Wb-NE in Clades 2 (Fig. 2), i.e., mesic northern Israel, xeric southern Israel and Jordan, and Iran and Turkey, highlighting the evolution and ecological adaptation of wild barley in the Fertile Crescent. However, Tibetan wild and some cultivated barleys (Clade 1) could be distinctly separated from the Near East wild barleys (Clade 2) (Fig. 2). STRUCTURE analysis showed similar results. Here, ΔK was used to estimate the optimum number of populations (Fig. S1). The four estimated populations were as follows: Wb-NE, cultivated barley (EA-2, C3, and C4), Wb-T (*1-ii*), and some Tibetan wild barley, as well as all Chinese six-rowed cultivated

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JX271894, JX271895, JX271896, and JX271897).

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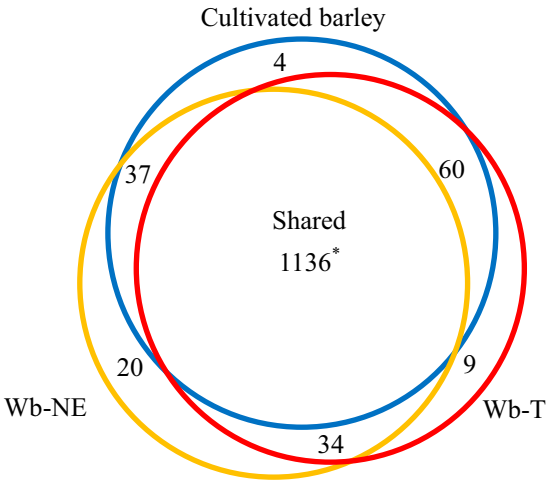

Fig. 1. Overlapping of polymorphic markers for cultivated barley and wild barley from the Near East (Wb-NE) and Tibet (Wb-T) in a Venn diagram. *There are 1,136 shared polymorphic DArT markers for all of these 3 groups, and 9 markers show no polymorphism within each population.

barley (C-6). Moreover, Wb-NE and Wb-T (*1-ii*) groups could be distinctly detected in the estimated populations when k was 3 and 4 or larger, respectively (Fig. S2).

Geographic Distribution of Wild and Cultivated Barley. To test the concept of polyphyletic origin of cultivated barley, we merged a dataset of representative barley germplasm in the Mediterranean basin (13) and used STRUCTURE to analyze the optimal population for cultivated and wild barleys. ΔK was plotted for increasing the number of K value determined by STRUCTURE analysis of 294 barley accessions and 419 DArT markers. The combined data also revealed four estimated populations according to ΔK (Fig. 3). Similarly, Wb-NE, Wb-T (*1-ii*), and Wb-T (*1-i*) were grouped in the different estimated populations (Fig. S2; k = 4). The mean membership coefficient (Q) of the four estimated populations in each prior subgroup of wild and cultivated barley was calculated based on all individuals in each subgroup. All of the prior subgroups were mapped to their respective original areas and presented with pie charts according to mean Q values (Fig. 3).

Wb-NE (blue) showed significant genetic division from Wb-T (*1-ii*) (yellow) and Wb-T (*1-i*) (red) (Fig. 3). The Eastern Mediterranean and Turkey cultivars, together with Wb-NE, were assigned to population 3 (POP3) and showed membership probability of 89.9% and 66.9%, respectively, thus confirming previous reports that the Near East Fertile Crescent is a primary center of barley origin and domestication (1, 2, 7). Interestingly, Chinese hulless and six-rowed barley (C-6) was only located on the subclade together with Wb-T (*1-i*) (Fig. 2) and shared no common ancestors with other wild barley in POP1 (Fig. 3), indicating that this kind of barley was domesticated in the Tibetan Plateau and its vicinity.

The probability for assignment of Wb-T (*1-i*) to POP1 was as high as 79.3%, and Wb-T (*1-i*) and Wb-T (*1-ii*) shared common ancestors (Fig. 3). The East Asian two-rowed (EA-2) cultivar was assigned to POP4 with Wb-T (*1-ii*) (Fig. 3), with membership probability of 65.3%, and showed a membership probability of 28.7% with Wb-T (*1-i*). Hence, we supposed that part of the genome of East Asian two-rowed cultivars may have originated from Tibetan wild barley. Surprisingly, the northern Mediterranean two-rowed cultivars, mainly from Germany, Holland, Sweden, and Denmark, were assigned to POP4, with a membership probability of 83.2%. Turkey cultivars showed 66.9% and 28.8% probability of membership with Wb-NE and Wb-T (*1-ii*), respectively, indicating that these accessions arose from the admixture of the two groups.

Genetic Diversity at Four Gene Loci Among Wild Barley. The SNPs detected in the sequencing part of four genes (*Dhn1*, *Isa*, *CBF3*, and *CBF4*) were used to validate the results obtained by DArT markers in genetic division between the wild barley populations. There were 691 bp of upstream regions in the *Dhn1* genes and 573, 710, and 684 bp of the coding sequence of *Isa*, *CBF3*, and *CBF4*, respectively (Table S1), which were deposited in the GenBank database. These four genes were involved in 89 mutations out of 2,661 bp, where there were 12 and 48 private mutations in Wb-T and Wb-NE, respectively (Tables S1 and S2 and Dataset S1). The results showed significant difference between the two wild barley populations in DNA divergence (Fig. S3). Moreover, the higher level of nucleotide diversity among Wb-NE (Table S1 and Fig. S3) suggested a diversifying selection of wild barley in the Near East Fertile Crescent. Cluster analysis exhibited a large genetic diversity of wild barley, and a clear clade of Wb-T (*1-ii*) with a bootstrap value of 57% was detected (Fig. S4), thus supporting the results of cluster (Fig. 2) and STRUCTURE (Fig. S2) derived from the 1,309 DArT markers.

Discussion

SNP markers have been widely used for studies of genetic diversity and evolution (9, 14, 15), but sequence information is required for marker development. DArT, which is based on DNA–DNA hybridization without sequence information, may provide repeatable high-throughput multilocus dominant biallelic markers (22, 23). In this study, we used both SNP and DArT markers. The current results showed that Tibetan wild barley distinctively diverged from the Near East wild barley (Figs. 2 and 3 and Figs. S2–S4), which may be attributed to ecological adaptation and climatic and geographic divergence of the two regions, cold versus hot. *Hordeum* originated around 12 Mya, and *H. spontaneum* and *H. bulbosum* shared the same H haplome, which diverged around 7 Mya (10). Therefore, *H. bulbosum* was used as an out group. According to Nei (27), the maximal path on the tree (Fig. 2) is between *H. bulbosum* and Gairdner (a modern cultivar) with a genetic distance of 0.734. We assumed that the split of Wb-NE and Wb-T occurred at the point of divergent time (DT) (Fig. 2), with the genetic distance of 0.444 between DT and *H. bulbosum,* and of 0.290 between DT and Gairdner. Thus, we estimated that the divergence time of *H. spontaneum* from the Near East and Tibet is around 2.76 Mya. Our results confirmed that there were at least two major wild barley populations, one from the Near East, rarely found at places with altitudes over 1,500 m (1, 2), and another one in the Tibetan Plateau, with an altitude over 4,000 m.

Table 1. Distribution of 903 mapped markers on seven barley chromosomes

Chromosomes	1H	2H	3H	4H	5H	6H	7H	All
Number of markers	108	174	154	47	128	121	171	903
Length (cM)*	216.6	210.7	209.1	183.2	238.9	169.0	179.2	1,406.7
PIC value	0.395	0.370	0.380	0.374	0.377	0.394	0.393	0.383

DArT marker locations were presented by Wenzl et al. (23) and Zhou et al. (26).
*The chromosome length is shown according to Zhou (26).

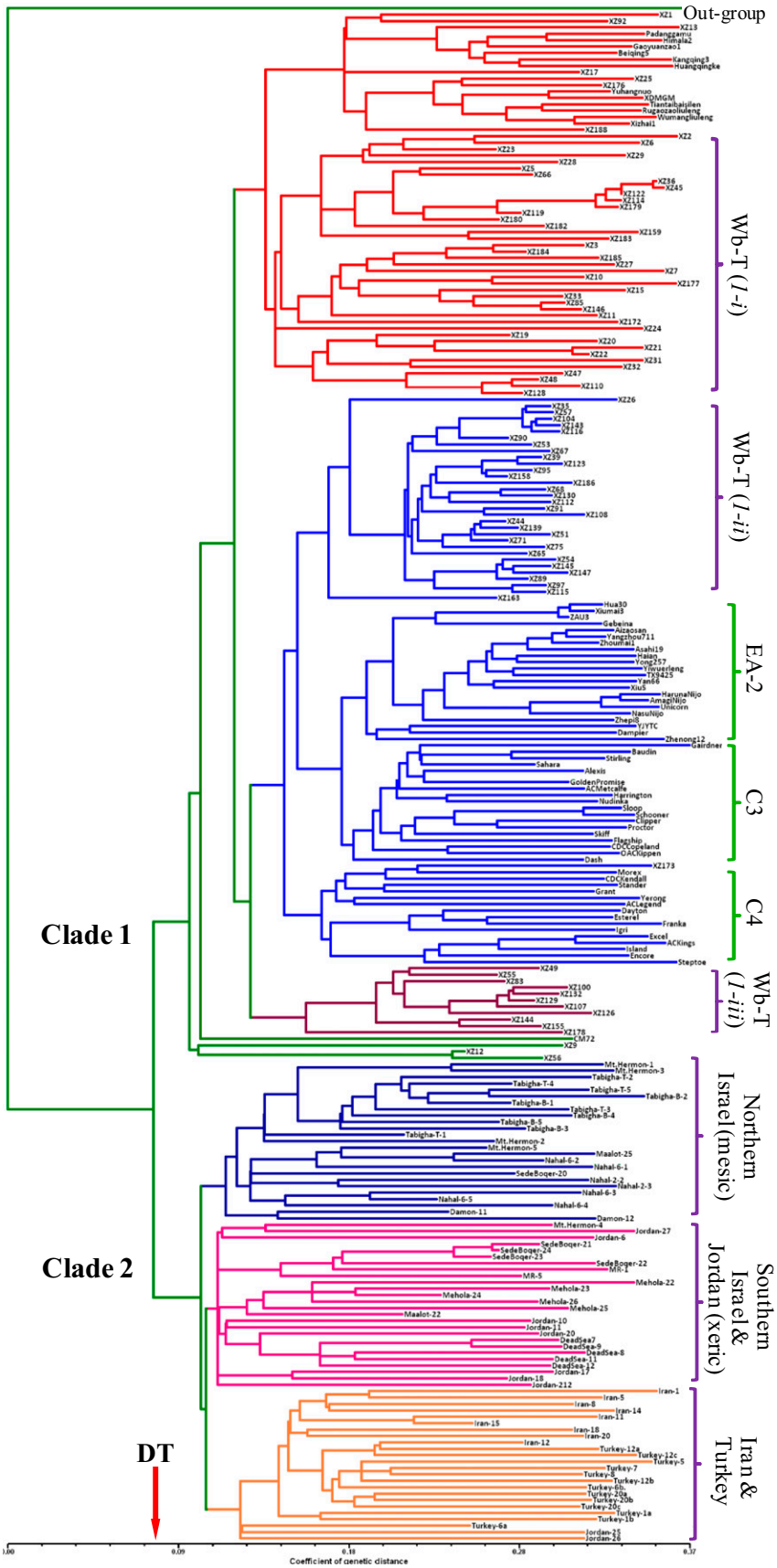

Fig. 2. Phylogenetic tree (neighbor-joining) of 238 barley accessions based on 1,309 DArT markers. Each accession is denoted as a vertical line in six colored subclades. Out group: *H. bulbosum*. Details of subgroup are described in Tables S3–S5. DT, divergent time between *H. spontaneum* in Tibet + *H. vulgare* and *H. spontaneum* in the Near East (Clade 1 and Clade 2).

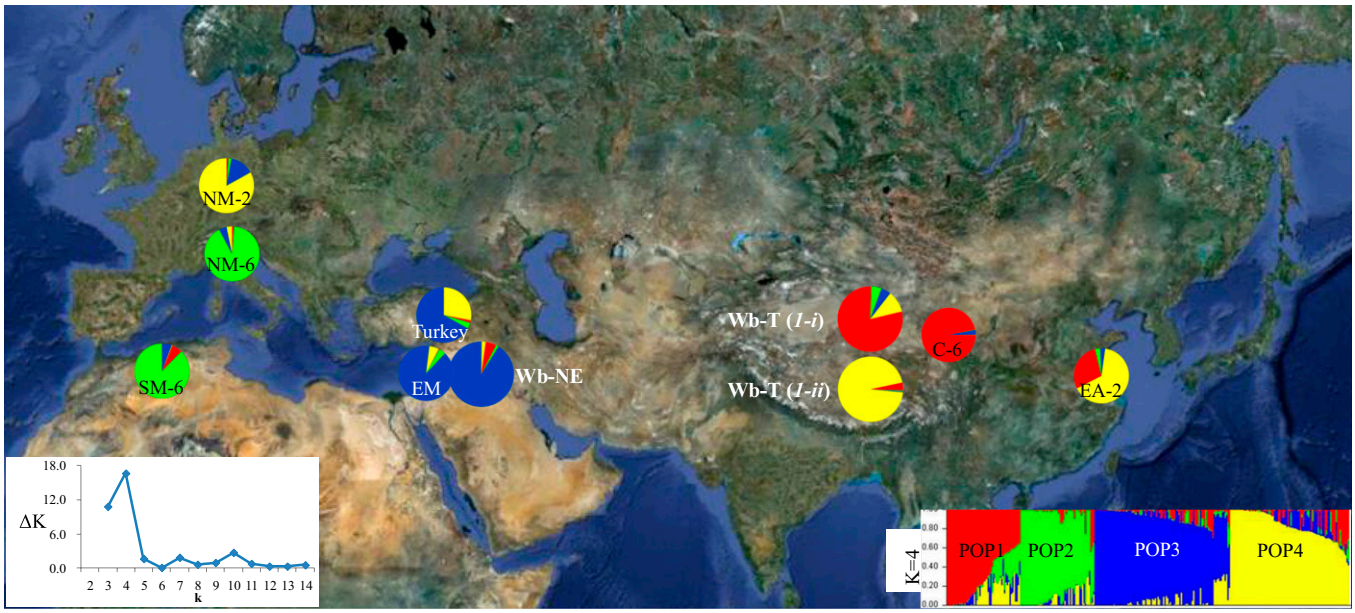

Fig. 3. Geographic distribution of wild and cultivated barley. The estimated probabilities of POP1 (red), POP2 (green), POP3 (blue), and POP4 (yellow) origin for each prior subgroup are calculated by an average membership coefficient (Q) of all accessions (the accession in each POP with Q value lower than 50% and the subgroup with an accession number less than 10 were discarded). POP1, Wb-T (*1-i*) ($n = 25$) + Chinese six-rowed (C-6) ($n = 12$); POP2, North Mediterranean six-rowed (NM-6) ($n = 25$) + Southwest Mediterranean (SM) ($n = 13$); POP3, Wb-NE ($n = 74$) + East Mediterranean (EM) ($n = 13$) + Turkey ($n = 10$); POP4, Wb-T (*1-ii*) ($n = 30$) + East Asian two-rowed (EA-2) ($n = 14$) + North Mediterranean two-rowed (NM-2) ($n = 28$). Wb-NE, wild barley from the Near East; Wb-T, wild barley from Tibet.

There was high genetic diversity in the accessions of Near East wild barley (Figs. S3 and S4). An alternative explanation is that Near East wild barley exists as populations, occupying large wild areas isolated from barley fields (1, 2, 7), whereas the Tibetan wild barley coexists as a weed with cultivated barley, wheat, and pea in the fields (19), harboring strong gene flows between wild and cultivated barley. This scenario may explain the close relationship between Tibetan wild barley and cultivated barley.

However, the origin of wild barley in the Tibetan Plateau remains to be revealed. In view of the fact that the uplift of the Himalayan Mountains began about 50 Mya (28), it may be assumed that Central Asia is the sole route for wild barley migration between the Near East and the Tibetan Plateau. Moreover, *Hordeum* may have evolved in Southwest Asia around 12 Mya and spread into Central Asia (29). Thus, the most likely scenario is that the Near East Fertile Crescent is a primary original center of wild barley. From this center, the wild barley migrated primarily to Central Asia and then spread upwards to the Tibetan Plateau from the north margin. Eventually, wild barley successfully adapted to harsh environments with extremely high altitude. During the formation of the alpine glacier in the Tibetan Plateau, some progenitors of Tibetan wild barley may have survived in lowlands, where extensive temperate refuges existed in subtropical latitudes (30). This may account partly for the low level of genetic diversity of certain genes in Tibetan wild barley (Figs. S3 and S4).

Our results indicated that some Chinese hulless six-rowed barleys had domesticated in the Tibetan Plateau and its vicinity (Figs. 2 and 3). The hulless barley, called "qingke," is widely cultivated and used as a staple food by Zang people (19). Moreover, the six-rowed cultivated barley from the Mediterranean basin was assigned in POP2 (green), thus confirming the report that the six-rowed phenotype originated at different times and in different regions independently (31). Intensive breeding has caused greater differentiation of these lines from wild ancestors and makes them genetically unique.

The East Asians and northern Mediterranean two-rowed cultivars, as well as Turkey cultivars, showed close genetic relationships (Fig. 3). Introgression between wild and cultivated barleys may occur frequently according to the individual's membership fractions (Fig. 3), which is enhanced by artificial activities such as germplasm exchange and breeding. Gene flow between Eastern and Western cultivars would occur through the Silk Road, and some Eastern barley might reach Turkey, an important transition station of the Silk Road between Rome and China (32). Because of their high cold tolerance and spring habit, these two-rowed accessions successfully spread into a large area of Europe, even into Nordic countries such as Sweden. Wild progenitors of barley proved to be wide in genetic diversity of abiotic and biotic stress tolerance or adaptation (1, 7). In conclusion, the wild and cultivated barleys in the Tibetan Plateau could provide elite germplasm for barley improvement in drought and cold tolerance, so as to extend into continental climates, northern latitudes, and also fight against unpredictable climate changes in the future.

Materials and Methods

Plant Materials. The wild barley populations used in this study were as follows: 75 wild barley accessions (*H. spontaneum*) from the Near East (Wb-NE) including Israel (43), Jordan (10), Iran (9), and Turkey (13) (Table S3), collected and characterized by Nevo and colleagues (3–7); and 95 wild barley accessions from Tibet (Wb-T) (Table S4), collected by Xu and colleagues since the 1960s from the extensive area of the Tibetan Plateau, stretching 1,500 km from the west to the east and 1,200 km from the south to the north, with altitudes ranging from 2,700 to 4,000 m (18, 19), and kindly provided by D. Sun (Huazhong Agricultural University, Wuhan, China). These wild barley accessions from Tibet included 57 two-rowed (*H. spontaneum*), 25 six-rowed (*H. agriocrithon*), and 13 intermediate accessions (18) (Table S4) according to their differences in morphology. It is commonly considered that *H. spontaneum* is a wild ancestor of barley, and *H. agriocrithon* is a hybrid between cultivated and wild barley as a result of introgression (18, 33, 34). However, the *H. agriocrithon* lines from Tibet and Israel showed different origins (8, 35).

In addition, we selected 68 cultivated barleys from Australia (12), Canada (10), the United States (6), Japan (4), Germany (3), the United Kingdom (2), Denmark (1), France (1), Algeria (1), and China (28, some of them are six-rowed hulless barley from Tibet and Xinjiang) (Table S5). The bulbous barley (*H. bulbosum*), used as an out group, was kindly provided by J. Wang (Zhejiang Academy of Agricultural Sciences, Hangzhou, China). All of those plant materials

are available in the barley programs of Zhejiang University and Zhejiang Academy of Agricultural Sciences, China, on request for scientific research only. Moreover, we merged a dataset of representative barley accessions in the Mediterranean basin and identified 419 DArT markers that had the same marker identification detected by Comadran et al. (13). The results of cluster analysis, using the 419 DArT markers, gave out the same groupings as reported by Comadran and colleagues (13, 14). Thus, we randomly selected cultivated barley within each prior subgroup, which almost completely covered all barley landraces and cultivars in the Mediterranean basin and East Asia.

Genotypic Analysis. Genomic DNA was extracted from young leaves of barley seedlings using a modified CTAB method. All of the DNA samples were sent to Diversity Arrays Technology (DArT P/L) in Australia for whole-genome profiling of DArT using the Barley PstI (BstNI) version 1.7 array (22). There are around 1,500 DArT markers, polymorphic in a wide range of barley cultivars, and 1,000 markers detected in wild barley accessions (http://www.triticarte.com.au/content/barley_diversity_analysis.html). From 1,576 reported markers, 1,309 polymorphic DArT markers were selected according to *P* value, of which 903 markers had been mapped to all of the seven chromosomes on the consensus map (23, 26).

Sequencing. We sequenced the genes of *Dhn1* (36), *Isa* (37), *CBF3*, and *CBF4* (38) based on the primers, PCR mixture, and program described by the references. All amplifications were performed on a DNA Engine Dyad thermal cycler (Bio-Rad). After purification of the PCR product, DNA sequencing was performed on an ABI 3730XL sequencer following the manufacturer's instructions (Applied Biosystems).

Data Analysis. PIC of each DArT marker was calculated according to the formula: PIC = 1 - $\sum (Pi)^2$, where *Pi* is the frequency of the i^{th} DArT marker (39). The matrix of DArT data was used for phylogenetic and population structure analysis.

We calculated genetic distance with NTSYSpc (version 2.10e) and developed a neighbor-joining tree according to a report by Nei (27). The population structure was analyzed based on genetically similar individuals with the STRUCTURE software (version 2.3.3), using an admixture model with five independent replicates of 100,000 Markov chain iterations (40). The value of ΔK was estimated based on the rate of change in the log probability of data between successive K values (41). The height of this value as an indicator of the strength of the signal was detected by STRUCTURE (41).

The obtained sequences were aligned using Mega version 5.0 (42), and unrooted phylogenetic trees were constructed using the neighbor-joining method (43). The confidence of each clade was estimated by bootstrap analysis using 10,000 pseudoreplicates. The properties of nucleotide, haplotype diversities, and recombination were evaluated with Dnasp version 5.0 (44).

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