Population Structure and Linkage Disequilibrium in Six-Rowed Barley Landraces from the Qinghai-Tibetan Plateau

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ABSTRACT
The genetic diversity, population structure, and extent of linkage disequilibrium (LD) were investigated at a genome-wide level in 255 six-rowed barley (Hordeum vulgare L. ssp. Vulgare) landraces from the Qinghai-Tibetan Plateau using 1264 polymorphic diversity array technology (DArT) markers. The mean polymorphism information content (PIC) of the DArT markers ranged between 0.008 and 0.500 with an average of 0.213. Bayesian, principal coordinate analysis, and phylogenetic analysis supported that six-rowed barley landraces from this region are divided into five distinct subpopulations centered on the regions of origin of the germplasm. The genetic distances calculated for all the individual pairs were geographically dependent, as revealed by Mantel tests (r = 0.35, P < 0.001) and spatial autocorrelation analysis. The LD values, expressed as r², declined with increasing genetic distance, and the same tendency occurred on each chromosome. In general, LD values were low (9.1% of adjacent markers; P < 0.001), and mean LD values across the whole genome decayed to below the critical r² of 0.22 after 3.32 cM. Approximately 19.0% of marker pairs (mean r² = 0.586) located on the same chromosome and 4.9% of pairs (mean r² = 0.318) located on different chromosomes were in LD (r² ≥ 0.22). Our results discerned relevant patterns of genetic diversity, population structure, and LD among members of a Qinghai-Tibet Plateau barley landraces panel proposed to be ‘typical’ of a target region or environment and have important implications for further studies on association mapping and practical breeding in high-altitude naked barley.

Cultivated barley is one of the oldest cultivated crops in the world (von Bothmer and Komatsuda, 2010; Zohary and Hopf, 2001; Zohary et al., 2012) and originates from wild barley (H. vulgare ssp. Spontaneum). Three regions, the Fertile Crescent (Badr et al., 2000; Harlan and Zohary, 1966; Zohary et al., 2012), Central Asia (Morrell and Clegg, 2007; Saisho and Purugganan, 2007), and Tibet (Dai et al., 2012; Nevo, 2013; Ren et al., 2013), have been suggested as the centers of wild barley domestication.
Barley is an ancient crop in China and has been known by the formal Chinese word Damai since 300 BC. A century ago, China was the world’s largest barley producer (Yang et al., 2010). Six-rowed hulless barley (H. vulgare L. var. nudum Hook. f.) was the earliest cultivated barley in China (Feng et al., 2006; Xu, 1982) and is known locally as qingke; it provides one of the staple foods for Tibetans inhabiting the Qinghai-Tibetan Plateau. Qingke is mainly used to produce the Tibetan staple food tsampa (known as zanba in Chinese) made from roasted flour mixed with salty butter tea and rolled into balls with the fingers (Newman and Newman, 2006; Saisho and Purugganan, 2007) and qingke liquor or beer. Almost all Tibetans have a daily diet that includes tsampa, buttered tea, qingke wine, and barley beer.

Six-rowed hulless barley cultivated in China is mainly distributed in the Qinghai-Tibetan Plateau and its vicinity, which covers the Tibet Autonomous Region and Qinghai Province, the Gannan Tibetan Autonomous Prefecture and Hexi Corridor Region of Gansu Province, the Ganzi Tibetan Autonomous Prefecture and the Aba Tibetan and Qiang Autonomous Prefecture of Sichuan province, and the Diqing Tibetan Autonomous Prefecture of Yunnan province. The Qinghai-Tibetan Plateau is referred to as ‘the roof of the world’ because of its high altitude. Crops that grow in these high-altitude regions (ranging from 2200 to 4750 m) are subject to difficult conditions, with drought, salinity, and cold being the major environmental stresses that impact survival and productivity. Six-rowed hulless barley landraces that grow in the Qinghai-Tibetan Plateau have high morphological diversity. Specific morphological features and agronomic traits include colorful seeds and extremely early heading times (45 d; Xu, 1982). Landraces have adapted well to the biotic and abiotic stresses of the extremely harsh habitat, making them ideal for providing an excellent germplasm to combat existing or upcoming stress threats (Du et al., 2011).

To further understand the domestication process and to facilitate its use in barley breeding programs, it is essential to study the genetic diversity and population structure of six-rowed hulless barley populations in Tibet and its vicinity (Dai et al., 2012; Feng et al., 2006; Nevo, 2013; Orabi et al., 2009; Ren et al., 2013; Wang et al., 2009). Although some six-rowed hulless barley landrace studies have taken place, these have so far only involved a small number of DNA markers and/or samples with limited geographical distribution (Dai et al., 2012; Feng et al., 2006; Li et al., 2004; Nevo, 2013; Ren et al., 2013; Wang et al., 2009; Zhang et al., 1992, 1994).

High-throughput DARt markers have been useful in studies of genetic diversity and population structure in barley (Bonman et al., 2011; Comadran et al., 2009; Dai et al., 2012; Mathies et al., 2012; Zhang et al., 2009). DARt analysis uses the barley Psf1 array, which comprises approximately 2500 clones known to be polymorphic in a wide range of Australian and European cultivars and in wild barley (Wenzl et al., 2004, 2006). The objectives of this study were (i) to investigate the population structure and genetic diversity at a genomewide level in six-rowed hulless barley landraces from the Qinghai-Tibetan Plateau using DARt markers, and (ii) to estimate the extent of LD in the barley genome as a first step towards performing association mapping.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

The six-rowed barley landraces investigated consisted of four hulled and 251 hulless types (Supplemental Table S1). These originated from the following Qinghai-Tibetan Plateau areas: Tibet (68 samples), Qinghai (65 samples), Gansu (85 samples), Sichuan (18 samples), Yunnan (12 samples), and Guizhou (two samples). Genomic DNA was extracted from young leaves using an improved cetyltrimethylammonium bromide method (Doyle and Doyle, 1990). The DNA samples were diluted at 50 to 100 ng μl⁻¹ and sent to Diversity Arrays Technology Pty. Ltd. (Yarralumla, Canberra, Australia) for whole-genome profiling of DARt using the Barley Psf1 (BstNI) version 1.7 array (www.triticarte.com.au; Wenzl et al., 2004).

Data Analysis

Polymorphism information content values were calculated for each DARt marker using the formula PIC = 1 – ∑(Pᵢ)², where Pᵢ is the proportion of the population carrying the ith allele (Botstein et al., 1980; Smith et al., 2000). Calculations were performed using the software Powermarker 3.25 (Li and Muse, 2005).

Three different methodologies were used to assess the population structure. 1. The Bayesian approach was used to determine the population structure using STRUCTURE 2.2 software (Pritchard et al., 2000). STRUCTURE was run three times independently with K ranging from 1 to 15, and with 10 iterations for each K, setting a burn-in period of 100,000 and burn-in length of 1,000,000 Markov Chain Monte Carlo iterations. For each run, STRUCTURE produced a Q-matrix listing the estimated membership coefficients for each accession in each subgroup. Statistical integration of data generated by STRUCTURE was performed using CLUMMP software (Jakobsson and Rosenberg, 2007). 2. Principal coordinate analysis (PCoA) was performed with the computer program NTSYS pc2.1 (Rohl, 2000) using a genetic similarity matrix based on Nei’s genetic similarity. Nei’s standard genetic distance assumes that genetic differences arise because of mutations and genetic drift, which differentiate it from other measures. 3. Phylogenetic analysis was conducted using the allele sharing distance matrix. The neighbor-joining (NJ) tree was constructed using NTSYSpc2.1 software (Rohl, 2000).

An analysis of molecular variance (AMOVA) was performed in ARLEQUIN v3.5 software (Excoffier and Lischer, 2010) to estimate the variance among populations and among accessions within populations. Population differentiation and significance were assessed by calculating pairwise Fₛₜ values for all population pairs also using ARLEQUIN v3.5. The significance of variance components was tested using 1000 permutations.
The correlation between genetic distance and geographic distance (measured in kilometers) was performed both through the Mantel test and spatial autocorrelation analysis among accessions with doubtful geographical origin. The Mantel test was implemented in the GenAlEx6.5 software (Peakall and Smouse, 2012; 999 permutations, significance level $P < 0.01$). A multivariate spatial autocorrelation method was used to detect the spatial genetic structure of the barley germplasm. The analysis was operated in the GenAlEx6.5 software (Peakall and Smouse, 2012) using the genetic distance and geographical distance matrices and generated a correlation coefficient $r$ across 27 selected spatial distance classes. A significant departure from the null hypothesis of no structure ($r = 0$) is obtained when a positive $r$-value falls outside of the 95% confidence intervals. To ensure accuracy of estimating the extent of the genetic structure, each distance class had approximately equal distance pairs and varied the number of distance classes (Peakall et al., 2003). The autocorrelogram was plotted as a function of geographic distance to elucidate the scale at which genetic and geographic distances are no longer correlated.

The LD between pairs of DArT loci mapped on the same and different chromosomes was evaluated using the software package TASSEL (Bradbury et al., 2007). The LD was estimated by squared-allele frequency correlations ($r^2$; Hill and Robertson, 1968). The $P$-values for the significance of pairwise LD among all possible pairs of mapped DArT loci were evaluated by TASSEL. A plot of $r^2$ values (significance $P < 0.001$) against the genetic distance in cM was created for the extent and distribution of LDs. The genetic distance for the pairs of loci mapped on different chromosomes was assigned as 200 cM. The decrease of LD with genetic distance was estimated by nonlinear regression (SPSS Statistics, version 18.0) following the methods of Remington et al. (2001). A critical value of $r^2$, as evidence of linkage, was derived from the distribution of the unlinked $r^2$. Unlinked $r^2$ estimates were square root transformed to approximate a normally distributed random variable. Then the parametric 95th percentile of that distribution was taken as a population-specific critical value of $r^2$, beyond which LD was likely to be caused by genetic linkage. The intersection of the loess curve fit to the syntenic $r^2$ with this baseline was considered as the estimate of the extent of LD in the chromosome (Breseghello and Sorrells, 2006).

**RESULTS**

**Polymorphisms of DArT Markers**

High-throughput DArT markers are repeatable dominantly biallelic markers that can be used for whole-genome profiling of barley (Alsop et al., 2011; Hearnden et al., 2007; Rodríguez-Suárez et al., 2012; Wenzl et al., 2004, 2006). For a biallelic marker, the minimum and maximum PIC values are 0 and 0.5, respectively. In this study, DArT analysis produced 1263 biallelic markers, with corresponding PIC values ranging from 0.008 to 0.500, with an average of 0.213. Of these 1263 DArT markers, 625 (49.5%) had known map locations. These included 116 with minimum allele frequencies (MAF) lower than 0.05 and three with missing data percentages >20%. Mean PIC values, calculated for each of the seven chromosomes, were found to be similar within six of the chromosomes (0.207–0.241). However, chromosome 4H had a significantly lower mean PIC value (0.116) and much less marker coverage than the other chromosomes (Table 1).

The 625 DArT markers provided an average genome coverage of 89 markers per chromosome (one marker per 2.25 cM), with a minimum of 37 markers per chromosome (4H; one marker per 4.95 cM) and a maximum of 125 (7H; one marker per 1.43 cM). Previous studies (Kraakman et al., 2004; Rostoks et al., 2005) have demonstrated that the genomewide coverage provided by the DArT markers in this study was dense enough for further association analysis.

**Population Structure**

Three approaches were used to partition the germplasm into subpopulations on the basis of the molecular polymorphism data collected. When considering the linkage between markers, a representative 122 DArT markers were used to investigate the genetic structures of the 255 barley landraces using the Bayesian approach implemented in the STRUCTURE 2.2 program. This subset was selected from the 625 mapped DArT markers on the basis of genetic distance, with approximately one marker every 10 cM.

The STRUCTURE program outputted the probability distribution $Pr(X|K)$, where $X$ represents the genotypes of all the examined accessions and $K$ the number of populations; a Q-matrix was generated for each $K$. Three methods were used to determine the optimal number of populations (Supplemental Fig. S1a). First, a plot of $\text{LnPr}(X|K)$ against $K$ was made for $K = 2$ to 15. In this plot, the value of $\text{LnPr}(X|K)$ rose steadily up from $K = 2$ to 15, and there were good duplications of the Q-matrices (measured as $r$; $r > 0.999$) for $K = 2$, 3, and 5. However, the replication of $\text{LnPr}(X|K)$ was poor for $K = 4$ and $K = 6$ to 15. Therefore, the analysis of $\text{LnPr}(X|K)$ did not obtain a clear optimal $K$.

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**Table 1. Distribution of 625 mapped diversity array technology markers on seven barley chromosomes.**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of markers</th>
<th>Length, cM $^1$</th>
<th>PIC value $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>85</td>
<td>216.6</td>
<td>0.230</td>
</tr>
<tr>
<td>2H</td>
<td>101</td>
<td>210.7</td>
<td>0.241</td>
</tr>
<tr>
<td>3H</td>
<td>95</td>
<td>209.1</td>
<td>0.221</td>
</tr>
<tr>
<td>4H</td>
<td>37</td>
<td>183.2</td>
<td>0.116</td>
</tr>
<tr>
<td>5H</td>
<td>92</td>
<td>238.9</td>
<td>0.223</td>
</tr>
<tr>
<td>6H</td>
<td>90</td>
<td>169.0</td>
<td>0.214</td>
</tr>
<tr>
<td>7H</td>
<td>125</td>
<td>179.2</td>
<td>0.207</td>
</tr>
<tr>
<td>All</td>
<td>625</td>
<td></td>
<td>0.216</td>
</tr>
</tbody>
</table>

$^1$ The chromosome length is given in accordance with Zhou et al. (2012).

$^2$ PIC, polymorphism information content.
value. Next, a plot was made for $\Delta K$ against $K$ (Supplemental Fig. S1b; Evanno et al., 2005). This plot revealed that there were two peaks, at $K = 2$ and $K = 5$, which suggested that the most likely value(s) of $K$ is 2 or 5. Finally, the relationship between genetic structure and phenotype was analyzed (Cockram et al., 2008; Jones et al., 2011). Phenotype data on the caryopsis structure (hulled or hulless) were converted into binary characters, and logistic regressions of phenotypes against the Q-matrices for $K = 2$ to 10 were performed in SAS package. In this type of analysis, the lowest value for Akaike’s information criterion (AIC) identifies the range of $K$ where the population structure is best correlated with phenotype. The AIC is a measure of the quality of a statistical model; when given a set of possible models for the data, the preferred model is the one with the minimum AIC value. For hulled and hulless, the lowest AIC value was found at $K = 5$ (Supplemental Fig. S1b). The combined results of the three methods suggested that the optimal $K = 5$.

The 162 accessions (63.5%) with membership coefficients of $Q \geq 0.8$ were assigned to one of five populations (POPs), while 93 accessions (36.5%) were classed as admixed (Fig. 1; Supplemental Table S1). The classification of the five populations was compared with their geographical distribution (Fig. 2). Population 1 mainly consisted of lines originating from the area along the Yarlung Zangbo River and its two tributaries (YLN) and Changdu region, Tibet, south Qinghai (the Yushu Tibetan Autonomous Prefecture), west Sichuan, and northwest Yunnan. The POP2 lines were from two regions in Tibet (YLN and Changdu). Population 3, which included all four of the hulled lines as well as hulless lines, was distributed from the margins or vicinity of the Qinghai-Tibetan Plateau (west Sichuan, northwest Yunnan, and the Hexi Corridor Region, Gansu). Population 4 mainly consisted of lines from the Hehuang Valley, and from the area between the Huangshui and Huang Rivers in Qinghai and Gansu, and its vicinity. Population 5 mainly included lines from south Gansu.

Principal coordinate analysis was also used to examine the population structure. Two two-dimensional and one three-dimensional scatter plot of the 255 barley genotypes are shown in Fig. 3. They exhibited a similar population stratification to that of the STRUCTURE analysis, and the first three principal coordinates accounted for 22.54%, 13.06%, and 8.29% of the genetic variance, respectively. For all assigned accessions, the first and second principal coordinates (PCo1 and PCo2) clearly separated POP1, POP2, and POP3 from POP4 and POP5 (Fig. 3a). The third principal coordinate (PCo3) further separated POP4 from POP5 (Fig. 3b). Population 4 and POP5 had the closest genetic relationship and were distinct from POP2 and POP3 (Fig. 3c). Although the genetic differences among the POPs generally matched the geographical distance, some individuals, identified as admixed in STRUCTURE, were placed in intermediate positions in the ordination (Fig. 3).

The Nei’s genetic distance coefficients ($d$), ranging from 0.000 to 0.584 (overall mean of 0.181), were calculated using NTSys 2.1e. The greatest distance ($d = 0.584$) was observed between the two Genotypes 705 (from Tibet) and 736 (from Sichuan). A NJ tree was constructed in accordance with $d$ (Fig. 4). The NJ tree could also be classified into five clear major branches. These were similar to the results from STRUCTURE and PCoA, but with a few exceptions treated as admixed accessions in the above two approaches. The composition of the five branches in relation to their geographical origin was similar to that described above. The closely related subpopulations POP4 and POP5 were clustered closer to POP1, which itself was closer to POP2 than POP3. Population 2 and POP3 represent two clearly separated branches in
admixed populations (from the Hexi Corridor Region and the vicinity of the Qinghai-Tibetan Plateau).

the NJ tree (Fig. 4). Vague small branches were observed between POP1 and POP2. These were mainly composed of lines that STRUCTURE analysis had identified as admixed populations (from the Hexi Corridor Region and the vicinity of the Qinghai-Tibetan Plateau).
Five populations were identified in the population structure analysis, and these were used to perform an AMOVA. Highly significant ($P < 0.001$) genetic variance resided within and among populations. The variance within populations accounted for a higher proportion (75.66%) of the total variance than that among populations (24.34%; Supplemental Table S2). This result indicates that accessions within populations are highly genetically differentiated in relation to accessions among populations. Moreover, the estimated fixation index ($F_{ST} = 0.2434$) was highly significant ($P < 0.001$) and indicates that this germplasm is highly differentiated.

Population pairwise $F_{ST}$ values of all pairs ranged from 0.14749 to 0.37816 and all pairs showed highly significant genetic differentiation ($P < 0.001$). The lowest and highest $F_{ST}$ values were observed between POP4 and POP5 and between POP2 and POP4, respectively (Supplemental Table S3).

Figure 4. Neighbor-joining phylogenetic tree of 255 barley landrace accessions from the Qinghai-Tibetan Plateau based on 1241 diversity array technology markers with <10% missing data. The Unweighted Pair Group Method with Arithmetic Mean cluster analysis was based on Nei’s genetic distances. Each accession is denoted as a vertical line in five colored subclades corresponding to the subgroups defined in the STRUCTURE analysis (POP 1 to 5).
Mantel tests between the genetic and geographical distance matrices were significant ($r^2 = 0.12$, $P < 0.001$; Supplemental Fig. S2a). The genetic relation tended to be more distant with increasing geographic distance. The same pattern was seen for the spatial autocorrelation analysis, which showed that within a distance class of 500 km, positive and significant $r$-values ($P < 0.001$) were detected. This indicates that the proximal landrace individuals were more genetically similar (i.e., related) than expected from a random distribution. The degrees of correlation, however, gently decreased, and there were negative and significant $r$-values over the 610-km distance class (Supplemental Fig. S2b). This result reveals that within 500 km, genetic similarity within accessions reduced and genetic dissimilarity increased over 610 km.

**Linkage Disequilibrium Analysis**

Of the 625 mapped markers, 116 presented a MAF below 0.05 and were expunged to prevent the detection of spurious LD. In addition, three markers were not used for LD analysis because >20% of their data was missing. The remaining 506 DArTs used for LD analysis were randomly distributed across the genome (Fig. 5). The squared-allele frequency correlation ($r^2$) for each pair of chosen markers was calculated. Markers with a significant $r^2$ of $P < 0.001$ were selected for use in subsequent analyses. Significant LDs were observed in 9.1% of the locus pairs and the average $r^2$ was 0.118 (Table 2). A critical value of $r^2$, beyond which LD is likely to be caused by genetic linkage rather than population structure, was determined according to the distribution of the unlinked $r^2$ estimates and fixed at 0.22 (Breseghello and Sorrells, 2006).

The relationship between $r^2$ and the genetic distances were assigned into five classes across the whole genome. The LD tended to decrease with increasing geographic distance. The level of LD decreased from an average $r^2 = 0.47$ when the distance was $\leq 3$ cM, to an $r^2$ level below the critical background value of 0.22 when the distance was >3 cM (Supplemental Fig. S3). This tendency was replicated within every chromosome except for chromosome 4H (Fig. 5). The proportion of markers with $r^2 \geq 0.22$ decreased from 66.41% to 7.41% within the first 10 cM, even though a large number of closely linked markers showed low levels of LD. Conversely, there were also a few contrasting examples where high $r^2$ values were observed between theoretically unlinked markers (>50 cM; Supplemental Fig. S4).

**Table 2. Percentage of loci pairs and mean $r^2$ in linkage disequilibrium (LD; $P < 0.001$, $r^2 \geq 0.22$) genomewide and for each barley chromosome (Chr.).**

<table>
<thead>
<tr>
<th>LD ($P &lt; 0.001$)</th>
<th>1H</th>
<th>2H</th>
<th>3H</th>
<th>4H</th>
<th>5H</th>
<th>6H</th>
<th>7H</th>
<th>Genomewide</th>
<th>Intra Chr.</th>
<th>Inter Chr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean $r^2$ of loci pairs $P &lt; 0.001$</td>
<td>0.252</td>
<td>0.155</td>
<td>0.153</td>
<td>0.430</td>
<td>0.155</td>
<td>0.155</td>
<td>0.223</td>
<td>0.118</td>
<td>0.187</td>
<td>0.101</td>
</tr>
<tr>
<td>Percentage of loci pairs in LD $r^2 \geq 0.22$</td>
<td>27.5%</td>
<td>17.7%</td>
<td>14.2%</td>
<td>65.0%</td>
<td>12.1%</td>
<td>13.6%</td>
<td>23.0%</td>
<td>7.6%</td>
<td>19.0%</td>
<td>4.9%</td>
</tr>
<tr>
<td>Mean $r^2$ of loci pairs in LD $r^2 \geq 0.22$</td>
<td>0.684</td>
<td>0.470</td>
<td>0.515</td>
<td>0.580</td>
<td>0.618</td>
<td>0.558</td>
<td>0.632</td>
<td>0.445</td>
<td>0.586</td>
<td>0.318</td>
</tr>
<tr>
<td>Mean distance (cM)† at which $r^2 = 0.22$</td>
<td>7.69</td>
<td>4.95</td>
<td>0.79</td>
<td>–‡</td>
<td>2.03</td>
<td>0.05</td>
<td>4.31</td>
<td>3.32</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

† For each chromosome, the average distance (cM) at which $r^2 = 0.22$ is indicated.
‡ For chromosome 4H, insufficient diversity array technology markers led to an incorrect result.
The $r^2$ values for intrachromosomal locus pairs ranged from 0 to 1, with an average of 0.187. Of these $r^2$ values, 19.0% exceeded 0.22 and had an average value of 0.586. The $r^2$ values for interchromosomal locus pairs ranged from 0.037 to 1, with an average of 0.101. Only 4.9% of the $r^2$ values exceeded 0.22 and had an average value of 0.318 (Table 2). The mean LD at which $r^2$ values fell below 0.22 was 3.32 cM (Table 2; Supplemental Fig. S4). The distribution of the $r^2$ values for the seven individual chromosomes is given in Table 2 and illustrates that different chromosomes had different LD levels. Next, the mean LD for each chromosome was explored. For chromosome 4H, the LD level was high and unreliable; this was because of the limited number of markers for this chromosome and their uneven distribution. When 4H is ignored, chromosome 1H had the largest LD decay distance, followed by 2H and 7H. Chromosomes 6H and 5H had the shortest LD decay distances at 0.05 and 0.79 cM, respectively. This suggests that chromosomes 1H, 2H, and 7H had been subjected to distinct natural and artificial systematic selection.

**DISCUSSION**

**Polymorphisms of Six-Rowed Hulless Barley Landraces from the Qinghai-Tibetan Plateau**

DArT markers that provided a relatively high-density genome-wide coverage have previously been shown to be useful for studies of genetic diversity and population structure in barley (Bonman et al., 2011; Comadran et al., 2009; Dai et al., 2012; Matthies et al., 2012; Wenzl et al., 2004, 2006; Zhang et al., 2009; ). However, this is the first study to exclusively assemble and analyze the germplasm of six-rowed barley landraces from the Qinghai-Tibetan Plateau using DArT markers and is expected to lay the foundations for future research.

The level of polymorphisms observed using DArT markers did not exceed those of previous studies. The mean PIC value of all the polymorphic markers from our samples was 0.213 (Table 1). This was much lower than the previously reported PIC values of 0.407 for the 192 landraces and cultivars from the Mediterranean basin (Comadran et al., 2009), 0.38 for 206 landraces likely to be from Ethiopia (Bonman et al., 2011), 0.379 for 170 wild barleys from the Tibetan Plateau and the Middle East (including Israel, Jordan, Iran, and Turkey), 0.533 for 68 world barley cultivars (Dai et al., 2012), 0.38 for 170 Canadian barley cultivars (Zhang et al., 2009), and 0.24 for 183 European cultivars (Mathies et al., 2012). While this low marker polymorphism is probably an indicator of the narrow regional or wild progenitor origin of hulless barley landraces from the Qinghai-Tibetan Plateau, it could be attributed to the original ascertainment bias from a DArT array that almost certainly did not include Qinghai-Tibetan Plateau barley landraces in its panel of polymorphism discovery genotypes (Moragues et al., 2010). As stated by Xia et al. (2005), however, these lower PIC values could prove beneficial owing to their potential for defining genetic structures.

**Population Structure in Six-Rowed Barley Landraces from the Qinghai-Tibetan Plateau**

In this study, Bayesian, PCoA, and phylogenetic analysis of polymorphic DArT markers consistently supported that six-rowed hulless barley landraces from the Qinghai-Tibetan Plateau are divided into five major subpopulations according to geographical distribution. All landraces of the subpopulation POP2 were distributed in Tibet (Fig. 2). Population 1 has a wider distribution range in the Qinghai-Tibetan Plateau. Although most of the landraces in POP1 were from Tibet, some came from nearby regions, including south Qinghai, west Sichuan, and northwest Yunnan (Fig. 2). In contrast, none of the POP5 landraces and only one landrace from POP4 was from Tibet. The two subpopulations were mainly distributed in the Hehuang Valley, the Gannan region, and the Hexi Corridor Region (Fig. 2). However, the phylogenetic analysis revealed that POP1, POP4, and POP5 were closely related to each other (Fig. 4). They could be further clustered, along with POP2, into a bigger clade. Tibet is recognized as one of the centers of domestication for cultivated barley (Dai et al., 2012; Feng et al., 2006; Nevo, 2013; Ren et al., 2013; Wang et al., 2009), with the region having a widespread distribution of wild six-rowed barley, an ancestor of six-rowed cultivated barley landraces (Åberg, 1938; Brücher and Åberg, 1950). In our study, there was a higher diversity of landraces from Tibet than observed in the other regions (Supplemental Table S4). Therefore, we speculate that POP1, POP4, and POP5 may belong with POP2 to a common ancestral population associated with the secondary center of domestication in Tibet.

The Ancient Tea-Horse Road (Feng et al., 2006) and the Ancient Tang-Tibet Road were two important roads for trade exchange between Tibet and outside regions. Historically, domesticated barley from Tibet may have first spread into the west Sichuan province and from there into surrounding areas, such as the Gannan region and northwest Yunnan via the Ancient Tea-Horse Road. In this study, landraces from Sichuan had the second highest PIC value rankings (Supplemental Table S4), and most of them (14 of 19) were grouped into POP1 (Supplemental Table S1). Furthermore, barley from Tibet may have spread into the Yushu region of Qinghai via the Ancient Tang-Tibet Road. The landraces from Yushu were also grouped into POP1, and their mean PIC value was lower than that of Tibet.

Although the accessions of POP4 and POP5 showed the closest genetic relationship in the phylogenetic tree, they had a different geographical distribution (Fig. 4). Most of the landraces in POP5 were from the Gannan region,
whereas most of those in POP4 were from the Hehuang Valley and the Hexi Corridor Region (Fig. 2). When the composition and hierarchical patterns of the POP4 population structure at values of K = 2 to 15 were analyzed, it appeared that landraces from the Hexi Corridor Region and the Hehuang Valley were derived from those of the Gannan region (Wang, unpublished data, 2012); this was supported by the phylogenetic tree data (Fig. 4).

Among the five subpopulations, POP3 had the widest geographical distribution range, covering the east margin area of the Qinghai-Tibetan Plateau. Its geographical distribution formed a crescent curve stretching thousands of kilometers. It was distantly isolated from the other four populations in the phylogenetic tree (Fig. 4). Meanwhile, this subpopulation, which included the four hulled and some hulless landraces, had the highest mean PIC values (Supplemental Table S4). The fact that POP3 contains some hulled barley may be significant because the peripheral geographic distribution makes it more open to outside (non-Tibetan) influences. It is not clear whether this is a Qinghai-Tibetan population that has drifted somewhat from its ancestral composition or a Central Asian or Middle Eastern-derived population (Badr et al., 2000; Morrell and Clegg, 2007; Zohary et al., 2012) that has adapted to the Qinghai-Tibetan Plateau environment, or a combination of the two.

Many of the landraces were recognized as admixed individuals (especially when the Q-value is near to 0.5; Fig. 1 and 3; Supplemental Table S1). These admixed individuals may be the result of introgression between landraces derived from different regions (Fig. 1 and 3). Artificial activities, such as germplasm exchange, can bring landraces naturally distributed in different regions into contact in the same fields; this is a prerequisite for the occurrence of introgression. The existence of germplasm exchange was confirmed by the fact that the landrace pair 42 and 170 had the same name, Dulihuang, but with slight genetic divergence (d = 0.016). In fact, 42 and 170 were from different collection points (42 was collected in Menyan County, Qinghai Province and 170 was collected in Hezuo City, Gansu Province in the 1950s). It is well known locally that the landrace Dulihuang originated from the Gannan region of Gansu Province and was introduced into the Hehuang Valley, Qinghai Province. This indicates that there was gene flow due to germplasm exchange and genetic divergence due to adaptation and selection.

Five distinct geography-driven subpopulations were derived from the analysis of population structure (Fig. 2), which was further confirmed by landscape genetic analysis. Mantel tests between the genetic and geographical distance matrices were significant (r = 0.35, P < 0.001). The spatial autocorrelation analysis between geographical and genetic distances reflects a continuous and constant reduction in the correlation coefficient r-values, from positive to negative (Supplemental Fig. S2). The boundary of the core region of each subpopulation is clear within 500 km and significantly eroded over 610 km, on average. Such a trend is characteristic of a typical strong selection along the geographical or environmental gradients accompanied with admixture or introgression (Sokal et al., 1989). For further analysis of spatial genetic structure in the present landraces panel, the correlation between genetic distance and ecogeographical and climate factors need to be taken into consideration.

**Linkage Disequilibrium**

The nonrandom association of alleles at different loci is known as LD. It plays a central role in association analysis, where it determines the number and density of markers required for whole-genome scans and the accuracy of the mapping (Comadran et al., 2009, 2011). In this study, the barley consensus map (Alsop et al., 2011; Hearnden et al., 2007; Mayer et al., 2012; Rodríguez-Suárez et al., 2012; Wenzl et al., 2006) was used to determine the positions of the 506 DArT markers used for LD analysis. The mean r² of the LD values higher than 0.22 extended up to 3.32 cM, and the average marker density of the genome was 89 markers per chromosome (one marker per 2.25 cM). This suggests that there is sufficient marker density for a whole-genome association scan. Indeed, previous studies (Comadran et al., 2009; Kraakman et al., 2004; Rostoks et al., 2005) have reported successful association mapping in barley using average marker densities of between 33.7 and 116 markers per chromosome.

LD has been shown to decay faster with increasing genetic map distance in wild relatives and landraces than in modern cultivated genotypes. This is because the genes of wild barley and landraces have not been subjected to the strong directional selection forces frequently used in plant breeding (Caldwell et al., 2006; Hamblin et al., 2010; Morrell et al., 2005). In contrast, LD extends over large linkage distances (sometimes over 50 cM) in some regions in related elite cultivated germplasms (Kraakman et al., 2004; Rostoks et al., 2005). In our sample of 255 landraces, the levels of LD were relatively low, with 9.1% of locus pairs showing highly significant statistical correlations at P < 0.001, 16.1% at P < 0.01, and 25.3% at P < 0.05; the general LD decayed steeply with map distances over 3.32 cM. The percentage of locus pairs in significant LD slightly exceeded that found between loci in a sample of 337 landrace accessions (about 22%, P < 0.05; Rodríguez et al., 2012). It was also more than that between loci in a sample of 25 accessions of the wild subspecies (H. vulgare ssp. spontaneum) from across its range (about 15%, P < 0.05; Morrell et al., 2005). Our LD decay distance is more than the 1 cM (r² ≥ 0.2) detected in 188 Tibetan wild barleys using 1125 DArT markers (L. Qiu, personal communication, 2013). The current LD decay distance is approximately similar to previous results of 3.2 cM in 192 landraces and cultivars.
(Comadran et al., 2009), 3.0 cM in 377 landraces (Rodriguez et al., 2012), but lower than 3.5 cM in an 86-sample subset of six-rowed barley cultivars (Zhang et al., 2009). Thus, our result of intermediate LD decay distance, lower than modern cultivars but higher than wild barley, is in agreement with previous molecular studies and basic cognizance in barley. When considering the higher LD level than that detected in 188 Tibetan wild barleys (L. Qiu, personal communication, 2013), we feel it is a consequence of barley landraces being subjected to distinct natural or artificial selection pressures found at the harsh multiple environments of the Qinghai-Tibetan Plateau.

Gene pools undergoing domestication experience dramatic changes in allele frequencies due to genetic drift or selection, and some allelic combinations may be lost. This leads to a generation of new, more extensive LDs (Hamblin et al., 2010). Previous studies have demonstrated that patterns of LD between a priori genes closely linked to a polymorphism of interest may be subjected to distinct selection pressures and recombination histories in the population (Caldwell et al., 2006; Comadran et al., 2009). This suggests that some chromosomal regions of ancestral barley populations, especially some regions of 1H, 2H, and 7H (Table 2, Fig. 5) in our landraces, which have high LDs in domesticated populations, were generated by artificial and natural selection processes in the harsh environments of the Qinghai-Tibetan Plateau during thousands of years of cultivation. Several important domestication- and adaptation-related genes are located on these three chromosomes; these include Ppd-H2 (photoperiod response) on 1H (Pourkheirandish and Komatsuda, 2007); Eam1 (early maturity; Wenzl et al., 2006), vrs1 (six-rowed spike; Tanno et al., 2002), and Ppd-H1 (photoperiod response; Wenzl et al., 2006) on 2H; and Vrn-H3 (vernalization response; Skinner et al., 2006; von Zitzewitz et al., 2005) and nud (naked caryopsis; Taketa et al., 2008) on 7H.

In this study, the average $r^2$ value at the $P < 0.001$ significance level was 0.187 at the intrachromosomal level and 0.101 at the interchromosome level. However, the $r^2$ values at $P < 0.001$ of the subpopulations tended to be much higher than for the total sample set and exceeded 0.5 at both the intrachromosomal and interchromosomal levels. This indicates that subpopulations had higher LDs that decayed more slowly across the entire genome. The main reasons for such high levels of LD were sample size and the high levels of similarity between accessions within every subpopulation (Comadran et al., 2009). This was an indicator that in subpopulations LD was probably caused by genetic linkage and not population structure. This conclusion indicates that the population stratification we had determined was suitable for our investigation.

Given the marker technologies developed for barley, the availability of extensively sampled wild-type, landrace, and modern cultivar gene pools and the considerable comparative phenotypic data collected from controlled field trials conducted over many sites and seasons (Waugh et al., 2009), ongoing studies on the species continue to present a useful model for the investigation of the evolution, adaptation, and spread of one of the world’s most important crops (Wenzl et al., 2006). The combining of population genomic data from landrace and natural populations, with information from field trials, provides a powerful approach to identify the genes responsible for adaptive phenotypes, quality traits, and agronomic characteristics (Waugh et al., 2009). In conclusion, the landrace barleys in the Qinghai-Tibetan Plateau could provide elite germplasm for barley improvement in the aforementioned areas. This investigation of population structure and LD is the basis for further genome-wide association studies, and for the identification and introgression of favorable exotic genes.

Supplemental Information Available
Supplemental information is included with this article.

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References
Hordeum vulgare


