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RESEARCH ARTICLE

Genetic and epigenetic variations in barley (*Hordeum vulgare* L.) using SSR and MSAP approaches

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Abstract Barley (*Hordeum vulgare* L.) is the fourth largest cereal crop in the world with extensive adaptability in diverse environments. There is increasing evidence that epigenetics contributes to plant evolution and adaptation. However, epigenetic variation in barley and their correlations with genetic variation remains largely unknown. In this study, we investigated epigenetic and genetic diversity in 48 Tibetan wild distribution (TWD) barley accessions, 24 Chinese cultivars (CC), and 24 foreign cultivars (FC) using DNA methylation-sensitive amplified polymorphism and simple sequence repeats. We found a relatively high level of epigenetic ($I = 0.639$ and $h = 0.450$) and genetic ($I = 0.637$ and $h = 0.446$) diversity in barley, and a significant correlation between epigenetic and genetic variation in barley ($R^2 = 0.160$, $P < 0.001$). No differences in genetic variation were observed among TWD, CC and FC populations. However, the TWD population had significantly higher epigenetic diversity ($I = 0.607$ and $h = 0.424$, $P < 0.001$) than both cultivated populations (FC: $I = 0.584$ and $h = 0.402$; CC: $I = 0.544$ and $h = 0.372$). Also, the TWD population had significantly higher average hemi-methylation (20.35%) and full-methylation (25.37%) levels than both cultivated populations (FC: 16.13% and 20.98%, respectively; CC: 15.98% and 18.95%, respectively). This study has provided valuable information on DNA methylation variation across diverse barley accessions and indicated that epigenetic variations might be another factor contributing to barley diversity.

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Introduction

Barley (*Hordeum vulgare* L.) is cultivated globally across a wide range of environments from Qinghai-Tibet Plateau to sub-sea level alluvial plain. It is used for feed, brewing malts, and human consumption (Mascher et al. 2017). Barley was domesticated ten millennia ago; the subsequent extensive migration provides striking evidence of its adaptation to different environments (Dawson et al. 2015). Wild barley is found in a wide range of environments with good tolerance to abiotic factors (Russell et al. 2016; Zhang et al. 2017).

Genetic variation is a major factor contributing to plant adaptation (Henderson and Salt 2017). Significant efforts have been made to explore genetic differentiation among barley populations (Fu and Peterson 2011; Hua et al. 2015; Morrell et al. 2013). An AFLP-based genetic diversity investigation in European barley accessions found a correlation between the level of genetic variability and degree of salinity tolerance (El-Esawi et al. 2018). Exome sequencing revealed that sequence variation contributed to range-wide eco-geographical adaptation in barley (Russell et al. 2016).

Numerous studies have demonstrated that epigenetic variation is another major factor regulating plant growth and influencing plant evolution and adaptation (Alakärppä et al. 2018; Kawakatsu et al. 2016; Latzel et al. 2013). Cytosine DNA methylation is an important epigenetic modification of nuclear DNA, which plays a crucial role in plant adaptation (Alakärppä et al. 2018; Kawakatsu et al. 2016; Latzel et al. 2013). In *Arabidopsis*, DNA methylation diversity increases the productivity and stability of *Arabidopsis* populations (Latzel et al. 2013). Natural DNA methylation variation is strongly correlated with local adaptation in *Arabidopsis* and Scots pine (Alakärppä et al. 2018; Kawakatsu et al. 2016).

DNA methylation-sensitive amplified polymorphism (MSAP) can detect genomic cytosine methylation in the 5'-CCGG-3' sites based on a pair of isoschizomeric restriction enzymes with different sensitivities to site-specific cytosine methylation. The MSAP method has been used widely to investigate DNA methylation variation in plants (Groot et al. 2018; Liu et al. 2018; Ma et al. 2018; Xie et al. 2017). An MSAP analysis detected adaptive epigenetic differentiation between upland and lowland rice (Xia et al. 2016). In another study, MSAP analysis detected little genetic but abundant DNA methylation differentiation in *Laguncularia racemosa* individuals from salt marsh and riverside locations (Lira-Medeiros et al. 2010). Some clonal plants have also been detected with high DNA methylation variations (Wang et al. 2019).

Barley has been proposed as a suitable model for studying adaptation to environmental changes (Dawson et al. 2015; Hill et al. 2019). The high level of genetic variation in barley may be one reason for its excellent adaptation to environments. Studies in *Arabidopsis* (Kawakatsu et al. 2016) and rice (Takata et al. 2005) have revealed clues between DNA methylation and domestication or adaptation. However, DNA methylation variation in barley remains unclear.

Because of its very high altitude, the Qinghai-Tibetan Plateau, also called 'the roof of the world,' is characterized by its extreme environment. Tibetan hulless barley (*Hordeum vulgare* L., qingke) has been the major food grown over an extensive area in the Qinghai-Tibetan Plateau for at least 3500 years (Dai et al. 2012; Zeng et al. 2018). Numerous studies have focused on the origin and genetic differentiation of Tibetan hulless barley and its wild distributions (Dai et al. 2012; Gong et al. 2009; Wang et al. 2015; Zeng et al. 2018). However, DNA methylation variations in Tibetan wild distribution (TWD) barley accessions have been overlooked.

This study aimed to characterize the extent of DNA methylation variability in barley and to determine any specific DNA methylation variations in TWD barley. We evaluated DNA methylation and genetic variation in 48 TWD barley accessions and 48 cultivated barley accessions using MSAP and simple sequence repeats (SSR) approaches.

Materials and methods

Materials

A total of 96 barley accessions from three populations were used in this study (Table S1). The TWD population contained 48 wild barley accessions from Qinghai-Tibet Plateau. The Chinese cultivars (CC) population contained 24 cultivars collected from eight provinces in China. The foreign cultivars population (FC) comprised 24 barley cultivars from 12 other countries. Seeds of each barley accession were germinated in pots and grown in a growth chamber (Conviron Company, MB, Canada) with a 14 h/10 h and 22 °C/16 °C day/night light and temperature cycle. At least three leaves of each accession were randomly collected 10 days after germination. There were three biological replicates for each accession.

MSAP and SSR analysis

DNA of each sample was extracted using the CTAB method (Storchova et al. 2000). MSAP is a modified version of cDNA amplified fragment length polymorphism (AFLP) (Vuylsteke et al. 2007) that incorporates *Bst*YI/*Hpa*II/*Msp*I as restriction enzyme combination. *Bst*YI (cut 5-'RGATCY'-3; Thermo Fisher Scientific, Waltham, USA) was used as a rare cutter, replacing the *Eco*RI enzyme. The isoschizomers, *Hpa*II/*Msp*I (Thermo Fisher Scientific, Waltham, USA)—with differential sensitivity to cytosine methylation at the CCGG sites—were used as frequent cutters. Two pre-primers of *Bst*YI were designed with one differently selective nucleotide at the 3' end (*Bst*YI-C, *Bst*YI-T). The sequence information of adaptors and 20 combined selective primers are shown in Table S2. For the SSR analysis, 20 pairs of SSR primers were used to detect genetic variations (Table S2).

The PCR reactions for MSAP and SSR were performed following the procedures described in Xu et al. (2009) and Xu et al. (2017), respectively. The PCR products of MSAP and SSR were separated by electrophoresis using a Fragment Analyzer™ Automated CE System (AATI, Ankeny, USA) with the Quick Start Guide 96 Capillary DNF-900 dsDNA Reagent Kit, 35–500 bp (AATI, Ankeny, USA). Raw data of SSR and MSAP were calculated using PROSize version 2.0 software (AATI, Ankeny, USA). The SSR and MSAP data were transformed into a binary character matrix, using '1' and '0' to indicate presence and absence.

Data analysis

The SSR binary was used to investigate genetic diversity among the three populations. For MSAP analysis, the *Bst*YI/*Hpa*II and *Bst*YI/*Msp*I binary data were divided into four types: Type I represents non-methylation [sites with the presence of bands in both *Bst*YI/*Hpa*II and *Bst*YI/*Msp*I (1, 1)], Type II represents hemi-methylation [bands present in *Bst*YI/*Hpa*II, but absent in *Bst*YI/*Msp*I (1, 0)], Type III represents full-methylation [bands present in *Bst*YI/*Msp*I but absent in *Bst*YI/*Hpa*II (0, 1)], and Type IV represents uninformative methylation [absence of bands in both enzyme combinations (0, 0)]. For epigenetic variance analysis, we reconstructed a methylation-sensitive polymorphism (MSP) profile, scoring Type II and Type III as '1', and Type I and Type IV as '0' (Schulz et al. 2013).

The relative hemi-methylation (Type II/total types), full-methylation (Type III/total types) and total methylation (Types II + III/total types) levels of each accession were calculated. Differences in DNA methylation levels between populations were identified with Wilcoxon's rank-sum test using R software (R Core Team 2013).

Genetic and epigenetic diversity analysis

The genetic or epigenetic diversity parameters, such as percentage of polymorphic loci (*PIC*), observed number of alleles (*Na*) and effective number of alleles (*Ne*), Nei's gene diversity (*h*), unbiased genetic diversity (*uh*) and Shannon's information index (*I*), were analyzed using GenAlEx version 6.5 (Peakall and Smouse 2012). Significant differences in genetic or epigenetic diversity parameters among three populations, were identified by Wilcoxon rank-sum test, and by the Kruskal–Wallis *H* test for all populations using R software (R Core Team 2013).

Molecular variance (AMOVA) was analyzed by GenAlEx 6.5 to investigate genetic and epigenetic variations (Φ_{ST}) among and within populations. Pairwise Φ_{ST} values were calculated from AMOVA to investigate genetic or epigenetic differentiation between two populations. Gene flow was calculated

as $Nm = (1 - \Phi_{ST})/4\Phi_{ST}$. Significance levels for AMOVA and pairwise AMOVA were based on 9999 permutations.

Principal coordinate analysis (PCoA) was performed with GenAlEx 6.5 based on Nei's genetic distances to investigate genetic or epigenetic distances among populations. Neighbor-joining trees for the 96 accessions were constructed with MEGA version 7 (Kumar et al. 2016) using Nei's genetic distances (1,000 bootstrap replicates) based on the SSR and MSP profiles. Genetic and epigenetic population structures between individuals were effected with a Bayesian clustering test, using STRUCTURE v 2.3.1 (Pritchard et al. 2000), to identify $K = 1$–10 clusters with an admixture model of correlated allele frequencies (burn-in period of 10,000 followed by 10,000 iterations) and repeated ten times for each K value. The Structure Harvester program was used to determine the most probable K-value using the ΔK method (Earl and Vonholdt 2012).

The correlation coefficient of genetic and epigenetic variation was calculated using the Mantel test (GenAlEx version 6.5, 9999 random permutations) based on epigenetic and genetic distance matrices of the SSR and MSP profiles.

Results

Genetic diversity and population structure

Forty alleles with high *PIC* were detected using 20 SSR primer pairs and visualized with PROSize software (Fig. S1). The lengths of the amplified fragments ranged from 101 to 220 bp. A high level of genetic diversity (h and I) were observed in the TWD ($h = 0.327$, $I = 0.482$), CC ($h = 0.376$, $I = 0.553$) and FC ($h = 0.395$, $I = 0.572$) populations (Table 1). However, no significant differences (Kruskal–Wallis H test for I, $P = 0.761$; h, $P = 0.748$) were detected among the three populations. AMOVA analysis revealed that the largest component of genetic variation was within each population (63.81%, Φ_{ST} = 0.362, and $P < 0.001$).

The pairwise AMOVA analysis showed significant genetic differentiation between populations (Table 2). A relatively low Φ_{ST} (0.187) and high Nm (1.087) were observed between the CC and FC populations (Table 2). However, a relatively higher genetic differentiation and lower gene flow were detected between TWD and the two cultivated populations ($\Phi_{ST} = 0.379$, $Nm = 0.410$ for TWD and FC, and $\Phi_{ST} = 0.429$, $Nm = 0.333$ for TWD and CC). These results confirmed a limited gene flow between TWD and the two cultivated populations.

The PCoA analysis was conducted on a genetic distance matrix using SSR data (Fig. 1a). The variation was explained by the first and second axes (26.11% and 10.76%, respectively). All accessions can be separated into the TWD and cultivated cluster by the second axes (Fig. 1a).

The neighbor-joining phylogenetic tree grouped the 96 accessions into the same clusters as the PCoA analysis (Fig. 2a), with a few CC accessions in the TWD cluster in the phylogenetic analysis. The population genetic structure analysis revealed a ΔK of 2 ($Ln\ P(K) = -2770.95$), dividing the 96 barley accessions into two clusters: TWD and cultivated populations (Fig. 3a, b).

Epigenetic diversity and population structure

Based on the 20 MSAP markers, 44,544 clear and reproducible bands, ranging from 100 to 500 bp (Supplementary Fig. 2), were produced for all the accessions and the average number of bands was 23.2 per primer. The TWD, FC, and CC populations had 458.01 (98.71%), 460.98 (99.35%), and 445.02 (95.91%) polymorphic loci, respectively. Epigenetic diversity (I and h) of the MSP profile is shown in Table 1. Notably, the TWD population had significantly higher epigenetic diversity than the FC and CC populations (Wilcoxon's rank-sum test, $P < 0.001$). The largest epigenetic variance was detected within populations (87.87%, $\Phi_{ST} = 0.121$ and $P = 0.001$) using hierarchical AMOVA.

Epigenetic differentiation between populations was also analyzed by pairwise AMOVA, with extremely low Φ_{ST} (0.004) and high Nm (63.580) between the CC and FC populations (Table 3). The TWD population had higher epigenetic differentiation and lower epigene flow than the two cultivated populations (Table 3).

The PCoA analysis also separated the accessions into the TWD cluster and cultivated cluster by axes 2 (Fig. 1b). A similar result was observed in the phylogenetic analysis (Fig. 2b). The epigenetic structure analysis

Table 1 Genetic and epigenetic diversity measures and characteristics of each population

Populations	*N*	*Na*	*Ne*	*PIC (%)*	*I*	*h*	*uh*
Genetic diversity							
Tibetan wild distributions (TWD)	48	1.950	1.593	95	0.482	0.327	0.331
Foreign cultivated (FC)	24	1.950	1.711	95	0.572	0.395	0.403
Chinese cultivated (CC)	24	2.000	1.669	100	0.553	0.376	0.384
Total	96	2	1.829	100	0.637	0.446	0.449
Epigenetic diversity							
TWD population	48	1.974	1.784	98.71	0.607A	0.424A	0.433A
FC population	24	1.987	1.720	99.35	0.584B	0.402B	0.419B
CC population	24	1.918	1.661	95.91	0.544C	0.372C	0.388C
Total	96	2	1.843	100.00	0.639	0.450	0.455

[a]Differences in the genetic or epigenetic diversity parameters between populations were analyzed by Wilcoxon's rank-sum test; different letters represent significant differences at the 0.01 level

[b]*N* Number of samples, *Na* Observed number of alleles, *Ne* Effective number of alleles, *PIC* Percentage of polymorphic loci, *I* Shannon's information index, *h* Nei's gene diversity, *uh* Unbiased genetic diversity

Table 2 Pairwise AMOVA analysis of genetic differentiation between populations

TWD population	FC population	CC population	
**	0.410	0.333	TWD population
0.379	**	1.087	FC population
0.429	0.187	**	CC population

[a]*ΦST* values below diagonal, *Nm* values above diagonal

[b]TWD, FC and CC populations represent Tibetan wild distributions, foreign cultivated and Chinese cultivated population, respectively

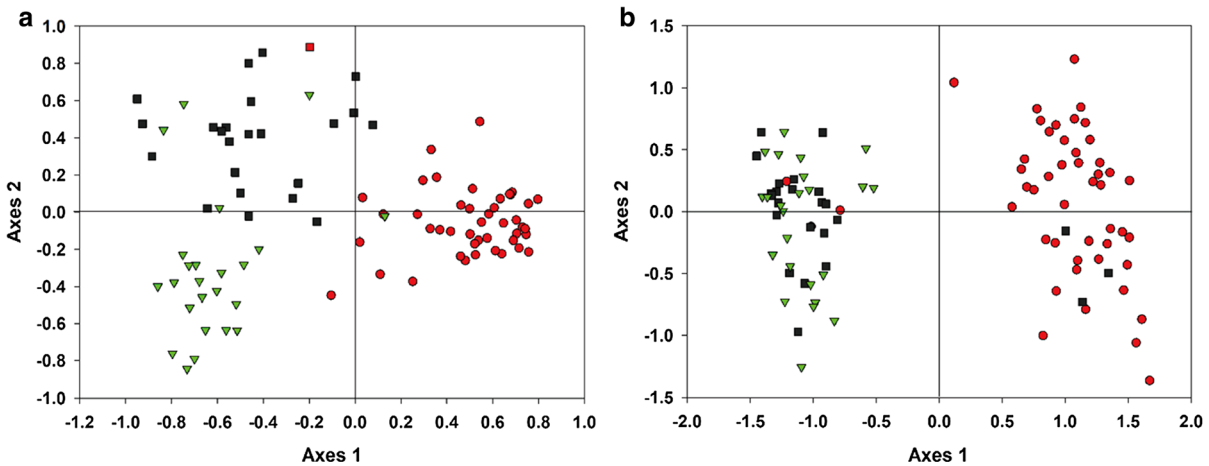

Fig. 1 Principal coordinates analysis showing the divergence within 96 barley accessions based on **a** genetic (SSR) and **b** epigenetic (MSP) markers. Red circles represent TWD individuals, black squares represent FC individuals, and triangles represent CC individuals. (Color figure online)

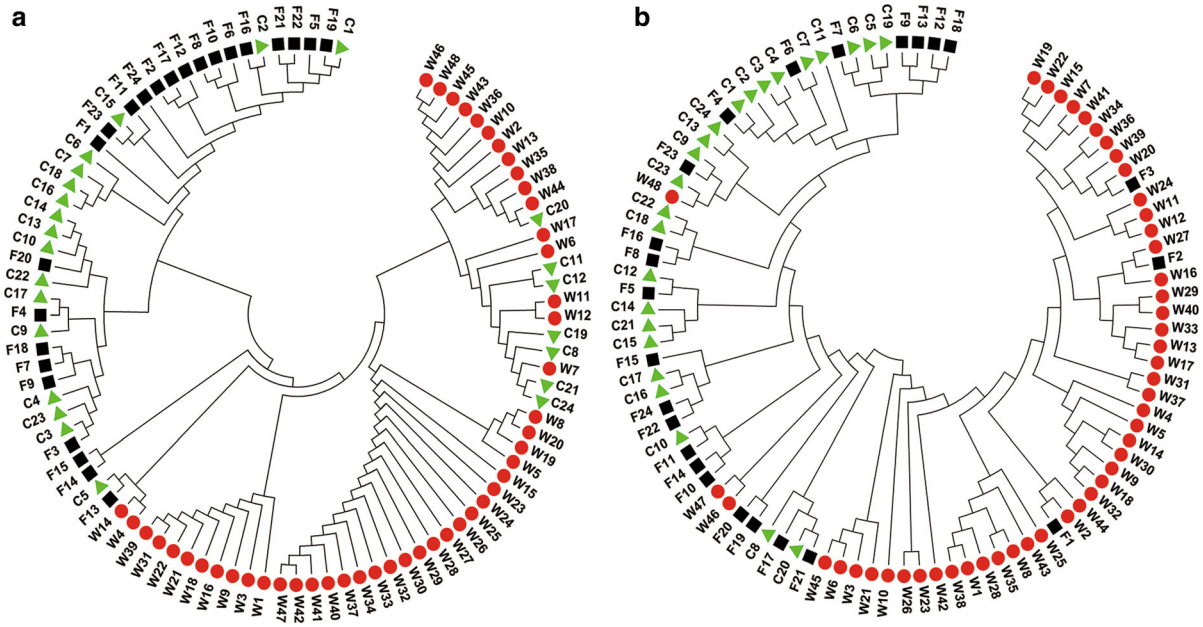

Fig. 2 Phylogenetic trees constructed from **a** SSR and **b** MSP data showing the relationships between the 96 barley accessions. W: TWD individuals, F: FC individuals, and C: CC individuals

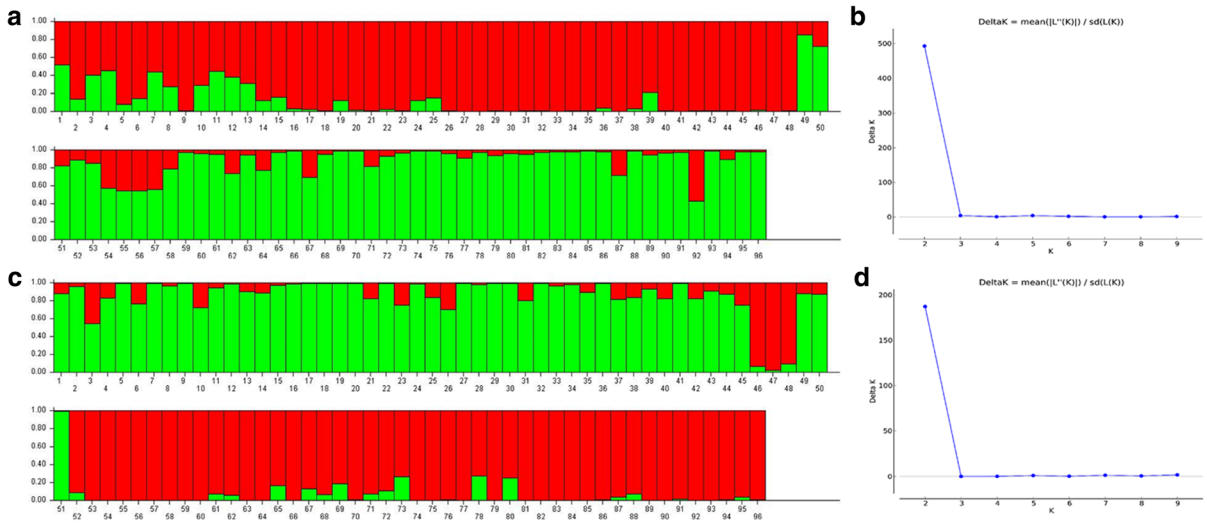

Fig. 3 Population structures analysis of genetic and epigenetic markers based on Bayesian clustering (A: SSR, C: MSAP) and the ΔK statistic (B: SSR, D: MSP)

also separated the 96 accessions into two populations ($\Delta K = 2$, $Ln\ P(K) = -51920.96$, Fig. 3c, d).

The Mantel test identified a significant correlation between epigenetic variation and genetic variation in barley ($R^2 = 0.160$, $P < 0.001$).

Relative genome DNA methylation levels in barley

Hemi-methylation levels ranged from 6.90% (Accession W45) to 29.96% (W10) in the TWD population, 10.78% (F15) to 25.43% (F3) in the FC population, and 9.27% (C2) to 22.63% (C22) in the CC population

Table 3 Pairwise AMOVA analysis of epigenetic differentiation between populations

TWD population	FC population	CC population	
**	1.718	1.286	TWD population
0.127	**	63.580	FC population
0.163	0.004	**	CC population

[a]*ΦST* values below diagonal, *Nm* values above diagonal

[b]TWD, FC and CC populations represent Tibetan wild distributions, foreign cultivated and Chinese cultivated population, respectively

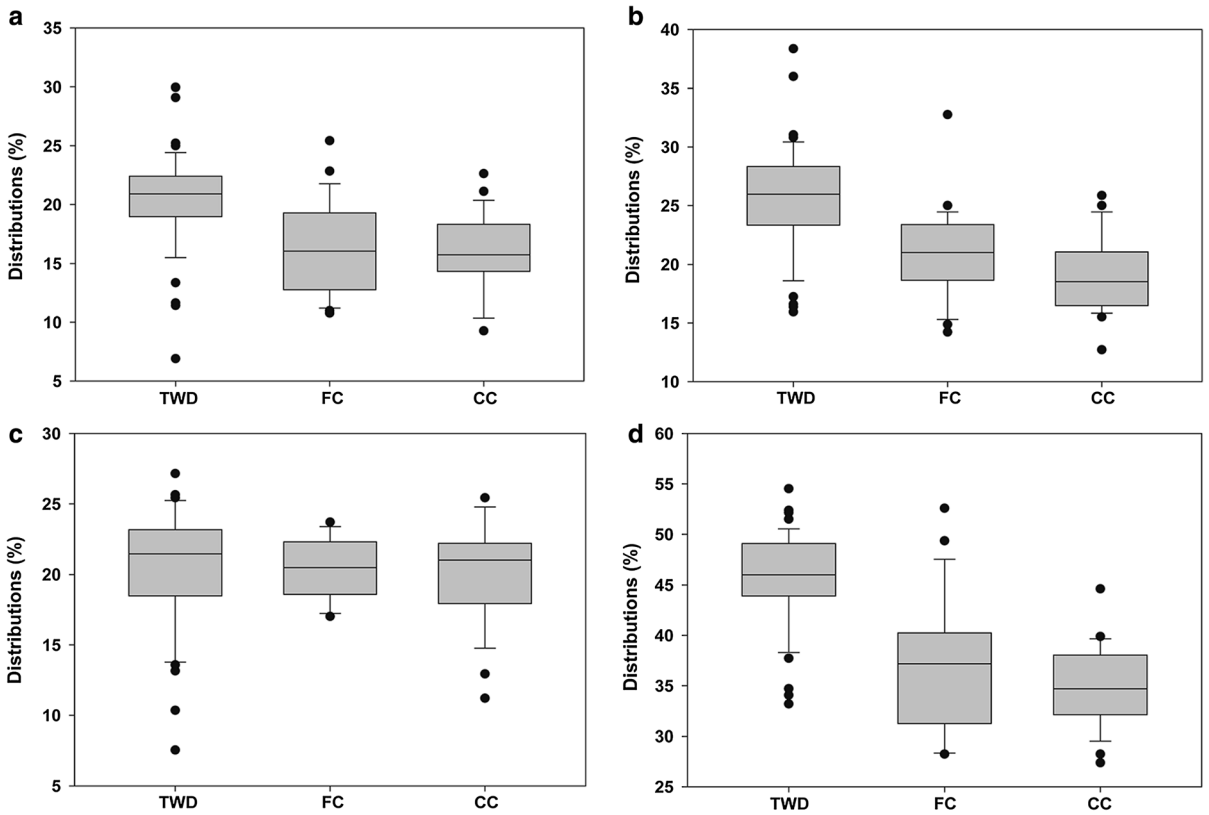

Fig. 4 Distribution of methylation levels within each population. **a** Hemi-methylation, **b** full-methylation, **c** non-methylation, **d** total methylation

(Fig. 4a). The TWD population had a significantly higher average hemi-methylation level (20.35%) than the FC (16.13%) and CC (15.96%) populations (Wilcoxon's rank-sum test, $P < 0.001$) (Fig. 4a).

Full-methylation levels ranged from 15.95% (W10) to 38.36% (W45) in the TWD population, 14.22% (F6) to 32.76% (F1) in the FC population, and 12.72% (C11) to 25.86% (C4) in the CC population (Fig. 4b). The TWD population also had significantly higher full- and total-methylation levels than the FC and CC populations (Fig. 4b, d; Wilcoxon's rank-sum test $P < 0.001$). It should be noted that MSAP could only detect hemi- and full-methylation at CCGG sites, and could not detect DNA methylation changes at the CG, CHG, and CHH sites.

Discussion

Barley (*Hordeum vulgare* L.) is the fourth largest cereal in the world and the model crop for understanding agronomic and physiological responses to climate change (Dawson et al. 2015; Hill et al. 2019). There is increasing evidence that genetic and epigenetic variation play crucial roles in plant adaptation to changing environments (Alakärppä et al. 2018; Alonso et al. 2016; El-Esawi et al. 2018; Feng and Jacobsen 2011; Henderson and Salt 2017; Kawakatsu et al. 2016; Kooke et al. 2015; Latzel et al. 2013; Richards et al. 2017; Russell et al. 2016; Takata et al. 2005). However, little is known about epigenetic variation in barley.

High level of DNA methylation polymorphism in barley

Studies have revealed a high level of allelic richness and variation in wild and cultivated barley and indicated multiple centers of domestication in cultivated barley (Dai et al. 2012; Morrell and Clegg 2007; Wang et al. 2015). This study revealed slightly lower levels of genetic diversity (h and I) than those in colored barley (Hua et al. 2015) and wild barley (Wang et al. 2015). The limited population size may be one reason for the lower genetic diversity in this study. Further genetic variation and relationships between populations could be revealed using high throughput markers or genome sequencing approaches (Hill et al. 2019). In this study, a relatively higher genetic/epigenetic differentiation and lower gene/epigene flow were detected among TWD and the two cultivated populations. The fragmented habitats of Tibetan wild distribution barley, geographical isolation of the Qinghai-Tibetan Plateau and self-pollinated may be part reasons for the limit gene exchange among TWD and the two cultivated populations (Dai et al. 2012; Guo et al. 2018; Ma et al. 2018).

Epigenetic changes play an important role in plant evolution under varied environments (Alakärppä et al. 2018; Feng and Jacobsen 2011; Kawakatsu et al. 2016; Kooke et al. 2015; Lira-Medeiros et al. 2010; Liu et al. 2018). A wide range of epigenetic diversity has been reported in different species. A higher level of epigenetic diversity was detected in this study (average $I = 0.639$, $h = 0.450$), relative to those in wild cherry ($I = 0.265$) (Avramidou et al. 2015), *Viola elatior* ($I = 0.230$) (Schulz et al. 2014), and maize ($I = 0.368$) (Roy et al. 2015). High levels of epigenetic diversity have been reported in rice ($I = 0.586$) (Xia et al. 2016), *Vitex negundo* var. *heterophylla* ($I = 0.534$) (Liu et al. 2018), commercial vineyards grape ($I = 0.555$) (Xie et al. 2017), *Scabiosa columbaria* ($I = 0.570$) (Groot et al. 2018), and *Prunus mume* ($I = 0.575$ and $h = 0.393$) (Ma et al. 2018). All these studies indicate that natural variation for DNA methylation is an important source of plant diversity.

DNA methylation variations in TWD

The TWD barley-specific DNA methylation variations were observed in this study. The TWD population had significantly higher epigenetic diversity than the two cultivated populations. Notably, the TWD population had significantly higher hemi- and full-methylation levels than the cultivated populations. Previous studies have found that most wild populations have similar or relatively higher epigenetic diversity levels than cultivated populations (Li et al. 2015). For example, wild ginseng has a higher level of genome DNA methylation than cultivated ginseng (Li et al. 2015). The TWD barley-specific DNA methylation variations may be due to the geographical isolation in Qinghai-Tibet Plateau. The epigenetic modification was easily affected by the environment and might lead to population convergence in similar habitats (Avramidou et al. 2015; Ma et al. 2018).

Correlation of genetic and epigenetic variation

The relationship between genetic and epigenetic variation is questionable (Avramidou et al. 2015; Liu et al. 2018; Schulz et al. 2014; Wang et al. 2019). Previous studies have reported that genetic and epigenetic variation are independent during plant evolution and demonstration (Avramidou et al. 2015). Our study identified a significant correlation between epigenetic and genetic variation, as reported by others for *Vitex negundo* var. *heterophylla* (Liu et al. 2018), *Viola elatior* (Schulz et al. 2014), and *Hydrocotyle vulgaris* (Wang et al. 2019), which suggests that epigenetic diversity is in part a downstream, subsidiary effect of genetic variation (Schulz et al. 2014; Wang et al. 2019). However, a recent population-level and genome-wide study in maize reported that DNA methylation variation is not tagged by genetic variation (Xu et al. 2019). Therefore, more

studies are needed to uncover the relationship between DNA methylation variation and genetic variation in plants.

Conclusions

In this study, a high level of epigenetic diversity was detected in barley, with the TWD population having significantly higher epigenetic diversity than the two cultivated populations. A significant correlation between genetic and epigenetic diversity was observed. Importantly, TWD population-specific DNA methylation variations were detected. These results suggest that epigenetic variation is a new source of variation in barley, which needs further investigation.

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Author's contributions YX and CL designed the experiments; BL, QG, WH, WZ, and GC conducted the research; YX and BL performed the statistical analyses; BL, XY, and CL wrote and finalized the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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