Pak. J. Bot., 50(4): 1379-1386, 2018.

HIGH HYPOMETHYLATION AND EPIGENETIC VARIATION IN FRAGMENTED POPULATIONS OF WILD BARLEY (*HORDEUM BREVISUBULATUM*)

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Abstract

The relationships between epigenetic variation and different environments have being received more attention, however, little is known of the population epigenetic changes by habitat fragmentation (HF). Our previous studies showed higher genetic diversity and differentiation in fragmented populations (FPs) of a wild barley species *Hordeum brevisubulatum*. In this paper, the effects on the population-epigenetic variation by HF were evaluated using marker methylation-sensitive amplification polymorphism (MSAP). 21 individuals of 5 populations were selected upon the data of AFLP (amplified fragment-length polymorphism) and SSAP (sequence-specific amplification polymorphism). Higher hypomethylation levels were in three FPs (average 3.72%), comparing with two unfragmented populations (UFPs, average 2.67%), and higher epigenetic diversities were also in FPs (0.292 of Nei's index) than in UFPs (0.249). Epigenetic variations were higher than genetic ones (AFLP and SSAP) within the populations, and individuals were dispersed more in cluster analysis using MSAP data; although most of the individuals belonging to a population were clustered into one group using all markers of MSAP, AFLP, and SSAP. The epigenetic molecular variation was lower between FP and UFP groups (3.09%) comparing with genetic ones (AFLP, 18.13%; SSAP, 13.87%), but higher among populations (23.25%) within the groups than genetic ones (AFLP, 12.69%; SSAP, 13.46%). In addition, epigenetic diversities were minimally correlated with genetic ones, and uncorrelated with geographical distances. Therefore, population epigenetic variation may be more sensitive than genetic one to the HF in wild barley.

Key words: *Hordeum brevisubulatum*, MSAP, Habitat fragmentation, Epigenetic variation, Genetic variation.

Introduction

Population genetic variation has been widely recognized as a primary source of adaptation to different natural environments. Habitat fragmentation (HF) generally reduces population genetic diversity, blocks gene flow, and increases genetic differentiation; the population subsequently loses adaptability to different habitats (Garcia-Fernandez *et al.,* 2013, Zhao *et al*., 2018). However, a recent series of studies suggested that epigenetic processes affect gene activities, and increase plant plasticity to various biotic and abiotic stresses (Rapp & Wendel 2005, Vandegehuchte & Janssen 2014), thus, epigenetic variance may be one of the core responding factors to ecology and evolution in natural populations. However, little is known of the epigenetic variance in fragmented populations (FPs).

Some rules exist in the natural occurrence of epigenetic variance. Firstly, DNA-methylation variation is more abundant than genetic one and specifically correlates with habitat environments (Chinnusamy & Zhu 2009, Schulz *et al.,* 2014, Medrano *et al.,* 2014). Higher epigenetic diversity was observed in *Laguncularia racemosa* growing in a salt marsh comparing with control plants (Lira-Medeiros *et al.,* 2010), and epigenetic variation among individuals of *Viola cazorlensis* was related to the amount of damage by herbivores (Herrera & Bazaga 2011). Then, some stress factors can change DNA methylation patterns. DNA hypomethylation patterns were discovered in plants growing in salt marsh (Lira-Medeiros *et al.,* 2010), drought (Tang *et al.,* 2014) and metal-contaminated (Kim *et al.,* 2016) conditions, but water stresses induced cytosine hypermethylation in pea (Labra *et al.,* 2002) and *Alternanthera philoxeroides* (Li *et al.,* 2013). Thirdly, epigenetic divergence is positively correlated with adaptive genetic divergence in wild violets (Herrera & Bazaga 2011) and *Onthophagus* (Snell-Rood *et al.,* 2013). Inversely, some studies showed that DNA-methylation variation is not associated with genetic variation (Herrera & Bazaga 2011, 2016, Avramidou *et al.,* 2015). Finally, epigenetic variances may reflect the changes in physiological and morphological traits, such as the flower morphology (Herrera & Bazaga 2010), adaptation history of cloned plants (Raj *et al.,* 2011), and heterophyll development (Herrera & Bazaga 2013). All those rules indicate that more debates are in the population epigenetic variations, and epigenetic variance may function as a straightforward adaptive source in the response of populations to the pressure of the HF.

Some high-throughput and high-resolution techniques were effective in model species (Schulz *et al.,* 2013). Methylation-sensitive amplification polymorphism (MSAP) (Reyna-Lopez *et al.,* 1997), determining DNA methylation in the abundant CpG sequences, is the most common technique used in epigenetic literature of non-model plants (Schulz *et al.,* 2013), because MSAP is an efficient, low-cost, and reliable method for detecting DNA methylation changes, and has been applied to evaluate the response of epigenetic variance to environmental perturbation in natural populations (Rapp & Wendel 2005, Schulz *et al.,* 2013, 2014, Preite *et al.,* 2015). Therefore, MSAP may be an appropriate marker to evaluate epigenetic variance of the FPs in wild barley.

Hordeum brevisubulatum (wild barley) was a dominant species in Songnen Plain (Kuo & Zhou 1980), where the environment was easily disturbed by human activities and formed fragmented habitats (Wang *et al.,* 2011). The HF severely blocks genetic communication between wild barley populations, consequently reducing the occurrence of rare alleles and resulting in higher genetic

heterogeneity (Guo *et al.,* 2016). Herein, some individuals belonging to five *H. brevisubulatum* populations were selected based on the results of amplified fragment-length polymorphism (AFLP) and sequence-specific amplification polymorphism (SSAP) data (Guo *et al.,* 2016). And the effects of HF on DNA-methylation pattern, epigenetic variance, and differentiation were evaluated using MSAP, and the possible correlations between epigenetic and genetic variances were also discussed.

Materials and Methods

Plant sampling and DNA extraction: A total of 21 individuals from five *H. brevisubulatum* populations were selected from 235 individuals belonging to nine populations, depended on the results of dendrogram and principle coordinate analysis (PCoA) by AFLP and SSAP markers (Guo *et al.,* 2016). The plants on each angle with high genetic distances among individuals in a population were selected. Among these plants, 14 individuals (SB1-1, SB1-10, SB1-12, SB1-13, SB1-24, SB1-25, DBS2, DBS11, DBS16, DBS23, DBS35, KZJ1, KZJ9, and KZJ13) belonged to three FPs, namely, SB1 (123°42.270′, l46°06.463′), DBS (123°39.726′, 44°51.960′), and KZJ (124°05.077′, 45°05.693′). Seven individuals (SS1-5, SS1-10, SS1-22, SS1-28, FY2-6, FY2-7, and FY2-10) belonged to two UFPs, namely, SS1 (124°00.610′, 47°14.782′) and FY2 (124°20.725′, 47°30.425′). The details of the five sites referred to Table 1 in Guo *et al.,* (2016). Genomic DNA was extracted from totally expanded leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Guo *et al.,* 2007) and was purified by phenolic extraction. The quality and quantity of DNAs were determined by gel electrophoresis and spectrometric assays.

MSAP amplifications: Genomic cytosine methylation in the 5'-CCGG-3' sites of different individuals was analyzed using MSAP marker described by Reyna-Lopez *et al.,* (1997). In brief, total genomic DNA (500 ng) was digested with two isoschizomers *Hpa*II (H) (20 U/μL, New England Biolabs, USA) and *Msp*I (M) (10 U/μL), and both combined with *EcoR*I (E) (20 U/μL), separately. These two isoschizomers can recognize the same sequence (CCGG), but differ in their sensitivity to DNA methylation patterns. The degested DNAs were subsequently ligated to H/E or M/E specific adapters (Table 1) using T4-ligase (Takara, Dalian, China), separately. Each PCR reaction in pre-amplification contained 0.3 μM [*H or M* + 1], 0.3 μM [*E* + 1] primers (Table 1), 1.0 U of rTaq DNA polymerase (Takara), 0.2 mM of each dNTP, and 2 μL of diluted restriction–ligation sample, 1× PCR reaction buffer (Takara) in a total volume of 20 μL. The amplification profile was one cycle of 72°C for 2 min, followed by 20 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and one final extension at 72°C for 3 min. The pre-amplification products were diluted for 50 times and then used as template for selective amplification. The selective amplification PCR was carried out using 2.5 μL of the diluted pre-selective amplifications, 0.1 μM each of two [*E* + 3] primers, 0.15 μM [*H*/*M* + 3] primers (Table 1), 0.5 U of rTaq DNA polymerase, 0.2 mM of each dNTP, and 1× PCR reaction buffer in a 20 μL volume. The amplification protocol was 1 cycle of 94°C for 2 min, 1 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min, followed by nine cycles of a 1.0°C decrease in annealing temperature per cycle, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 3 min. The amplifications were performed in a PTC-100 Thermal Cycler (The MJ Research Inc., Waltham, MA). Accordingly, 50 selected primer pairs were screened. Only the primer pairs with clear and reproducible bands were selected. Three independent amplifications were performed on the 21 individuals using the selected primer pairs (Table 1) to decrease the error rate among the different individuals within each treatment. Only bands with sizes ranging from 150 bp to 500 bp with sufficient intensity were scored to reduce the potential effect of size homoplasy (Vekemans 2002).

Analysis of MSAP data: The scored MSAP bands were transformed into a binary matrix, in which "1" indicates the presence and "0" indicates the absence of a band at a position. Banding patterns were classified into four types according to their cytosine methylation status based on the criteria of Salmon *et al.,* (2008) and Lira-Medeiros *et al.,* (2010). Non-methylated locus [bands were present in both sites of E/H and E/M, (1, 1)] designated as type A; hemimethylated locus at the external cytosine of the restriction site [bands were present only in sites of E/H (1, 0)] designated as type B; hypermethylated locus [bands were present only in sites of E/M (0, 1)] designated as type C; and non-informative locus [bands were absent in both profiles (0, 0)] designated as type D. Type D cannot discriminate between hypermethylation or nucleotide mutation.

The MSAP data were converted into a methylation-susceptible matrix, as described by Herrera & Bazaga (2011) using R program *msap* version 1.1.6 (Pérez-Figueroa 2013). To evaluate the population epigenetic variance, the loci of types B and C were scored "1", whereas the locus of type A was scored "0". The locus of type D was treated as "missing". The AFLP and SSAP matrices of "0, 1" of the 21 plants were extracted from the data of 235 individuals (Guo *et al.,* 2016), to interpret the possible relationships between epigenetic (MSAP) and genetic (AFLP and SSAP) changes by the HF. Retrotransposon *BARE-1* here was employed to evaluate the polymorphisms within or near its sequences in SSAP (Guo *et al.,* 2016, Kalendar *et al.,* 2011). Nei's gene diversity (GD) (1978), Shannon's information indexes (I) (Lewontin 1972) and Nei's genetic distance (1978) were estimated by software POPGENE (Yeh *et al.,* 1999). Epigenetic/genetic differentiation was assessed by genetic difference (F_{ST}) and molecular variance analysis (AMOVA) using ARLEQUIN 3.5 (Excoffier & Lischer 2010). The significance of variance components was obtained by nonparametric procedures using 1,000 random permutations. PCoA was performed using Nei's genetic distance with NTSYS (Rohlf 2000). The Bayesian-based software STRUCTURE (Falush *et al.,* 2007) was used to forecast the genetic and epigenetic structures of the five populations. The number of genetic cluster *K* from 1 to 5 was determined using a non-admixture model with independent allele frequencies between populations. The

burn-in period was 50,000 and delta *K* (Evanno *et al.,* 2005) was estimated by the true number of genetic groups with STRUCTURE HARVESTER (Earl & vonHoldt 2012). Instance by distance (IBD) and different significance analysis were determined by Spearman's non-parametric correlations (Hollander & Wolfe 1973), and significant difference was checked by *t* test using SPSS version 11.0 (SPSS Inc., Chicago IL). The geographical distances between populations were calculated using the formula of the great-circle distance between localities (http://www.movable-type.co.uk/scripts/latlong.html).

Results

Characteristics of primer pairs: A total of 456 loci and 167 polymorphic loci (36.6%) were generated from 21 plants using 12 MSAP primer pairs (Table 1), which were selected from 50 primer pairs. M4 had the least number of loci (21), whereas M12 produced the most number of loci (50) with 38 loci per primer pair. The matrices of 273 and 224 polymorphic loci of 21 individuals were extracted from the AFLP and SSAP datasets, respectively (Guo *et al.,* 2016).

Genomic DNA methylation changes of the FPs and UFPs: The locus status of DNA methylation was classified into four types (Table 2). Most of the loci were clustered into type A (non-methylated locus, about 80%), and slight lower (79.96%, average) was in three FPs comparing with that (81.08%) in two UFPs, indicating more DNA methylation changes or DNA mutations in FPs. Hemimethylated type B was significantly higher (t test, $p<0.05$) in the FPs (3.72%, average) than in the UFPs (2.67%), and similar result was in type D (not determined): FPs (10.32%), UFPs (7.62%) ($p<0.05$). However, the percentage of hypermethylated type C was markedly ($p<0.05$) lower in the FPs (5.99%) than in the UFPs (8.71%). Those showed that higher hypomethylation, and some of the undiscriminated nucleotide changes were possibly induced by the HF. Moreover, the modified DNA-methylation patterns might be involved in the population's adaptation to environment changes by the HF.

Table 1. Adaptors and primers used in MSAP analysis and characteristics of the selected primer pairs.

Type/Code	Sequences
Adapters	
*Eco*RI-adapter I	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adapter II	5'-AATTGGTACGCAGTC-3'
*Hpa*II/*Msp*I-adapter I	5´-GATCATGAGTCCTGCT-3´
*Hpa*II/*Msp*I-adapter II	5´-CGAGCAGGACTCATGA-3´
Pre-selective primers	
*Eco*RI +A	5'-GACTGCGTACCAATTCA-3'
*Hpa*II/*Msp*I+0	5´-ATCATGAGTCCTGCTCGG-3´
Selective primer combinations used in MSAP (Number of loci per primer pair produced / Number of polymorphic loci, percent of polymorphic loci (%))	
M1	*Eco*RI adapter+**AGG**-3´/*Hpa*II/*Msp*I adapter+**TCT**-3´ (32/19, 59.4)
M2	*Eco*RI adapter+**AGG**-3´/*Hpa*II/*Msp*I adapter+**TCG**-3´ (38/15, 39.5)
M3	*Eco*RI adapter+**ACA**-3´/*Hpa*II/*Msp*I adapter+**TCC**-3´ (38/17, 44.7)
M4	*Eco*RI adapter+**AGA**-3´/*Hpa*II/*Msp*I adapter+**TCC**-3´ (21/4, 19.0)
M5	*Eco*RI adapter+**AGC**-3´/*Hpa*II/*Msp*I adapter+**TTC**-3´ (49/17, 34.7)
M6	*Eco*RI adapter+**ACT**-3´/*Hpa*II/*Msp*I adapter+**TTG**-3´ (40/9, 22.5)
M7	*Eco*RI adapter+**ACA**-3´/*Hpa*II/*Msp*I adapter+**TCG**-3´ (43/14, 32.6)
M8	*Eco*RI adapter+**AGA**-3´/*Hpa*II/*Msp*I adapter+**TCG**-3´ (31/7, 22.6)
M9	*Eco*RI adapter+**AGC**-3´/*Hpa*II/*Msp*I adapter+**TCC**-3´ (42/17, 40.5)
M10	*Eco*RI adapter+**ACA**-3´/*Hpa*II/*Msp*I adapter+**TTC**-3´ (32/20, 62.5)
M11	*Eco*RI adapter+**AGG**-3´/*Hpa*II/*Msp*I adapter+**TTC**-3´ (40/10, 25.0)
M12	*Eco*RI adapter+**ACC**-3´/*Hpa*II/*Msp*I adapter+**TT**G-3´ (50/18, 36.0)
Total	(456/167, 36.6)

Table 2. The characteristics of DNA methylation patterns in 5 populations of *Hordeum brevisubulatum*.

Group	Population name	Type A		Type B		Type C		Type D		Total (%)
		Percent	Average	Percent	Average	Percent	Average	Percent	Average	
FPs	SB1	80.19	79.96	4.09	3.72*	6.54	5.99*	9.17	10.32*	1
	DBS	81.00		3.99		4.91		10.09		1
	KZJ	78.70		3.07		6.51		11.70		1
UFPs	SS1	80.59	81.08	2.74	2.67	8.22	8.71	8.44	7.62	1
	FY2	81.51		2.49		9.21		6.80		1

Type A: bands both present in a locus of a plant by E/H and E/M (1, 1); Type B: band only presents in one locus of a plant by E/H (1, 0); Type C: band only presents in one locus of a plant by E/M (0, 1); Type D: no band presents in one locus of a plant by E/H and E/M (0, 0). E: *Eco*RI, H: *Hpa*II, M: *Msp*I, FPs: fragmented populations, UFP: unfragmented populations.
*: Significant difference between FPs and UFPs, t text, $p<0.05$.

Table 3. The genetic and epigenetic diversity indexes of MSAP, AFLP and SSAP.

Group	Sample size	MSAP		AFLP		SSAP	
		GD	I	GD	I	GD	I
Fragmented	14	0.292	0.430	0.269*	0.409*	0.284*	0.429*
Unfragmented	7	0.249	0.366	0.176	0.264	0.194	0.292
Total	21	0.319	0.471	0.285	0.439	0.298	0.456

GD: Nei's gene diversity, I: Shannon's information index, FPs: fragmented populations, UFP: unfragmented populations.
*: Significant difference between FPs and UFPs t text, p<0.05

Table 4. The matrices of population genetic and epigenetic distances using marks MSAP, AFLP and SSAP, and the geographical distances between populations.

Population code	SB1	DBS	KZJ	SS1	FY2
MSAP					
SB1	0.000				
DBS	0.268	0.000			
KZJ	0.374	0.259	0.000		
SS1	0.285	0.257	0.318	0.000	
FY2	0.263	0.303	0.294	0.197	0.000
AFLP					
SB1	0.000				
DBS	0.120	0.000			
KZJ	0.154	0.074	0.000		
SS1	0.209	0.148	0.153	0.000	
FY2	0.236	0.157	0.168	0.056	0.000
SSAP					
SB1	0.000				
DBS	0.111	0.000			
KZJ	0.176	0.096	0.000		
SS1	0.188	0.142	0.168	0.000	
FY2	0.223	0.156	0.225	0.077	0.000
Geographical distance between populations (Kilometer)					
SB1	0.000				
DBS	62.203	0.000			
KZJ	31.601	42.460	0.000		
SS1	256.287	268.646	241.167	0.000	
FY2	308.376	327.189	295.644	69.521	0.000

Epigenetic diversity of the FPs and UFPs: Epigenetic diversity was established by Nei's gene diversity (GD) and Shannon's information indexes (I) (Table 3). Both GD and I parameters showed that the epigenetic diversity in the FPs was higher (GD: 0.292 and I: 0.430) than that in the UFPs (GD: 0.249 and I: 0.336). Those results were different to AFLP and SSAP data (Table 3), AFLP and SSAP indexes were significantly different between the UFPs and FPs ($p < 0.05$) and conforming to our previous data (Guo *et al.,* 2016), and the results also confirmed that the selected 21 plants could maximally mirror the population diversity of the five populations, similar indexes were also showed in Figs. 1, 2, and Table 5. Epigenetic diversity (measured by MSAP) indexes were higher than genetic ones in all populations. This finding implied that the HF in *H. brevisubulatum* population led to a high amount of epigenetic diversity or heterogeneity. Similarly, the epigenetic distances were significantly higher than the genetic ones ($p<0.01$; Table 4), indicating possible higher epigenetic variation among populations in *H. brevisubulatum*. However, no correlation between epigenetic and geographical distances ($r_{MSAP} = 0.06$, $p = 0.568$) was found using IBD analysis, which was contrary to the results of genetic IBD analysis ($r_{AFLP} = 0.711$, $p = 0.980$; $r_{SSAP} = 0.681$, $p = 0.977$). The higher p values might be the insufficient number of individuals, but the results still indicated that epigenetic variance was mainly related to habitat environments and not reflected the mass migration history of *H. brevisubulatum*.

Epigenetic differentiation of the FPs and UFPs: The higher indexes of the epigenetic/genetic diversity of the total populations (Table 3) and epigenetic/genetic distances (Table 4) indicated that epigenetic differentiation existed in and among populations. Hierarchical AMOVA confirmed this hypothesis (Table 5). Similar to genetic variance, a high percentage of epigenetic variance (73.67%) was observed within the populations, whereas 26.3% was observed among the populations and groups. However, only 3.09% variance was found between the FP and UFP groups, too lower than the genetic ones by AFLP (18.13%) and SSAP

(13.87%). However, epigenetic variance 23.25% was found among the populations within the groups and about two times than the genetic variance indexes of AFLP (12.69%) and SSAP (13.46%). Those results showed that the epigenetic variance of population is easily influenced by habitat environments. Meanwhile, the dispersal of the individuals in a population was larger in MSAP than those in SSAP and AFLP, although the majority of the 21 plants were classified into three groups using MSAP data by PCoA (Fig. 1a), similar with the results of AFLP (Fig. 1b) and SSAP (Fig. 1c). Moreover, the populations in the FP group were clustered into two sub-groups (SB1 was one sub-group). STRUCTURE analysis also confirmed those results when $K = 3$ (Fig. 2). However, epigenetic groups were sorted in a different order comparing with genetic groups. The red group in AFLP and SSAP included SB1 alone, whereas the red group in MSAP included SS1 and FY2. These findings were similar to the result of PCoA (Fig. 1), and indicated a minimal correlation between epigenetic and genetic variances in *H. brevisubulatum* populations. Thus, the epigenetic variance was mainly related to habitat environments, which probably promoted epigenetic differentiation in *H. brevisubulatum*.

Discussion

Significantly, HF impacts epigenetic variances of *H. brevisubulatum* populations, such as the higher genomic DNA hypomethylation levels, higher epigenetic diversity, and more differentiation. Thus, this paper might offer some novel opinions to study the influences of the HF on the evolution of the natural populations, and to protect the wild barley in the severely destroyed fragmented habitats.

The HF in Songnen Plain, mainly being caused by human activities in the last three decades (Wang *et al.,* 2011, Huang *et al.,* 2012), might result in a high pressure on *H. brevisubulatum* and led to changes of genomic DNA demethylation to some extent (Table 2). This phenomenon was also discovered in plants under various stresses, e.g., cold (Hashida *et al.,* 2006), drought (Li *et al.,* 2013, Tang *et al.,* 2014), salt (Lira-Medeiros *et al.,* 2010), and heavy metals (Kim *et al.,* 2016). For example, the transcriptome-level patterns were paralleled by the differences in genome-wide DNA demethylation in *Populus* under drought conditions (Raj *et al.,* 2011). The genomic DNA of the salt marsh plants were lower methylated, 14.6% of DNA methylation loci was observed from the total loci than that 32.1% from the riverside plants, (Lira-Medeiros *et al.,* 2010).

DNA methylation is usually associated with gene inactivation, whereas DNA demethylation results in gene activation (Schulz *et al.,* 2013). Thus, the higher DNA demethylation levels in FPs might activate some genes in wild barley. Generally, the transposon DNA sequences in plants are heavily methylated, and always occur within the gene promoters or transcribed regions within the transposons (Zhang *et al.,* 2006). The transposon *Tam-3* in *Antirrhinum* was activated by cold stress-induced hypomethylation (Hashida *et al.,* 2006). The promoter of the *colorless non-ripening* (*CNR*) gene, a component of the regulatory network that controls fruit ripening in tomato, is extensively methylated in all individuals that carry the *CNR* phenotype, but is largely unmethylated in the wild-type fruits (Manning *et al.,* 2006). Those results indicated that the HF might lead to higher genomic DNA hypomethylation levels in wild barley, DNA demethylation then activates some genes within transposons, such as retrontransposon *Bare-1*, used in SSAP analysis, *were* activated, and consequently led to higher genetic diversity detected by SSAP (Guo *et al.,* 2016). However, the relationships among population fragmentation, DNA methylation, activity of retrontransposon *Bare-1* and genetic variance need further investigation. Contrarily, pea (Labra *et al.,* 2002) and *A. philoxeroides* (Li *et al.,* 2013) under water stress showed cytosine hypermethylation. Thus, DNA methylation or demethylation may be the key factors in plants responding/adapting directly to the environmental changes. And the DNA methylation pattern changes of *H. brevisubulatum* might exhibit a similar strategy of re-activating some genes by demethylation, and adapt to the fragmented habitats.

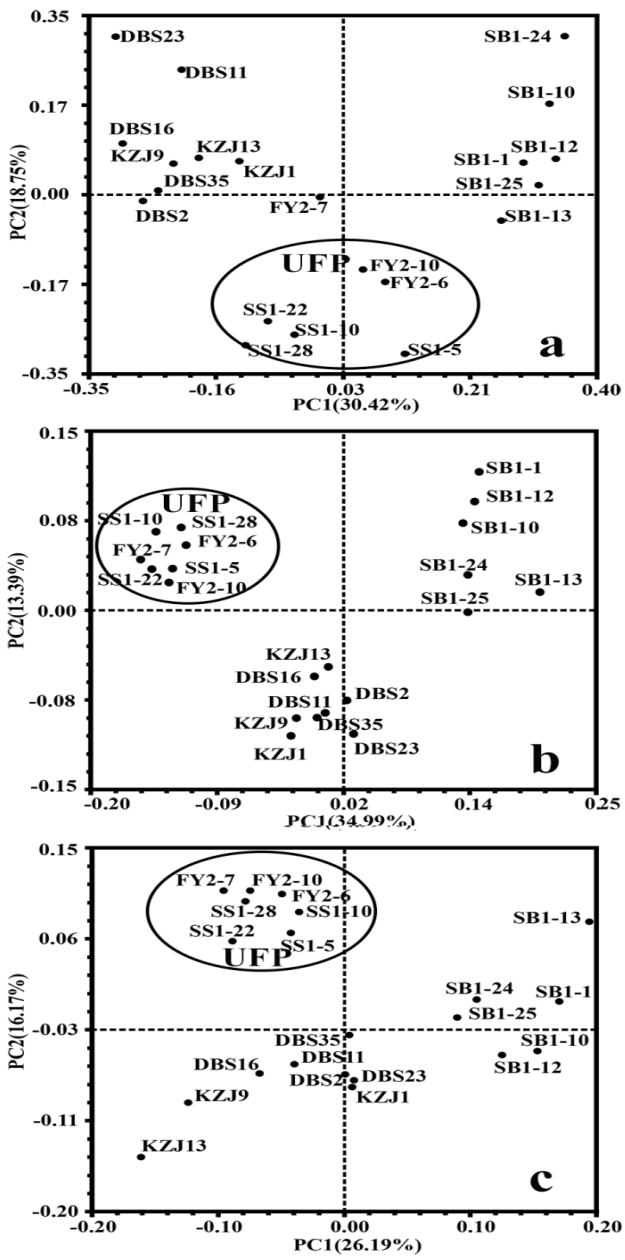

Fig. 1. Principle coordinates analysis (PCoA) using Nei's genetic distance matrixes of 21 *Hordeum brevisubulatum* individuals. a: MSAP, b: AFLP, c: SSAP. UFP: unfragmented population.

Table 5. Molecular variance analysis (AMOVA) of 5 populations of *Hordeum brevisubulatum*, and 5 populations were classed into fragmented (SB1, DBS, and KZJ) and unfragmented (SS1 and FY2) groups.

Source of variation	MSAP	AFLP	SSAP
Among groups	3.09%	18.13%	13.87%
Among populations within groups	23.25%	12.69%	13.46%
Within populations	73.67%	69.19%	72.67%
Fst	0.263	0.308	0.273
P	<0.01	<0.01	<0.01

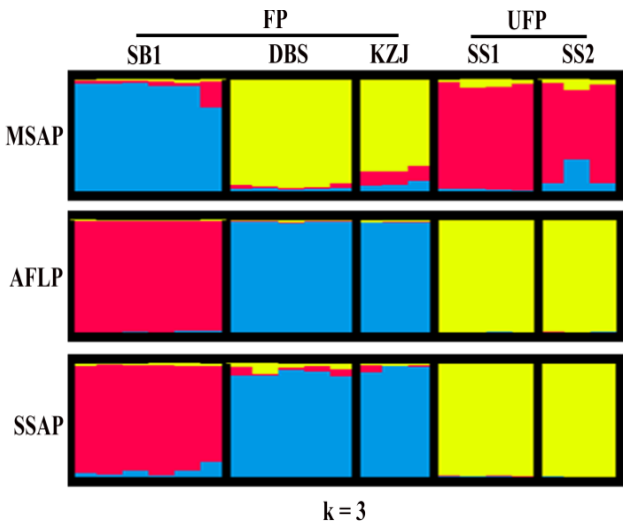

Fig. 2. District plots for STRUCTURE analysis of 5 *Hordeum brevisubulatum* populations based on MSAP, AFLP and SSAP datasets. FP: Fragmented population, UFP: Unfragmented population.

Previous studies have shown that epigenetic variation was higher than genetic variation within and between populations, our results demonstrated similar findings (Table 3), and epigenetic distance indexes were also significantly larger than genetic ones (Table 4). Similar results have been presented in white mangrove (Lira-Medeiros *et al.,* 2010), *V. cazorlensis* (Herrera & Bazaga 2010), and *Helleborus foetidus* (Medrano *et al.,* 2014). In dandelion, comparing with the control, a 30% change in polymorphic methylation-sensitive markers was observed when plants were exposed to different stresses (Verhoeven *et al.,* 2010). Those studies indicated that the HF may be as a stress factor that affects the epigenetic and genetic variance/heterogeneity in *H. brevisubulatum*. The changes in genomic epigenetic patterns may be response for a short time to stress pressures (Schulz *et al.,* 2014), such as bottleneck effect, which results in the decrease of genetic diversity with the increase of epigenetic diversities (Rapp & Wendel 2005, Furrow & Feldman 2014). However, the *H. brevisubulatum* populations in Songnen Plain have been severely fragmentized over the last 60 years, and such fragmentation might be sufficient to maintain the epigenetic equilibrium within populations, but insufficient for maintaining equilibrium of genetic variance. Generally, perenniality, outcrossing, and polyploidy of wild barley (Kuo & Zhou 1980) have been regarded as factors that contributed to the maintenance of high genetic diversity in FPs (Klank *et al.,* 2012). Such factors might lead to a rapid repair of methylation changes after epigenetic perturbance. Thus, *H. brevisubulatum* might buffer the excess epigenetic changes caused by the HF. However, this hypothesis should be confirmed further.

The epigenetic-molecular-variation indexes in *H. brevisubulatum* were specific to habitats/ populations/ treatments (Table 5), and similar results were in a wild barley species *H. spontaneum* (Sharma *et al.,* 2005) and other plants, such as natural *B. ermanii* plants growing in alpine tundra and subalpine areas (Wu *et al.,* 2013), plants treated with salt (Lira-Medeiros *et al.,* 2010), drought (Raj *et al.,* 2011, Li *et al.,* 2013, Tang *et al.,* 2014), and tissue culture pressure (Guo *et al.,* 2007, Shair *et al.*, 2016). Those findings agreed with that the correlation between epigenetic variance and habitat environments/ stress treatments was more than that between genetic variance and habitat environments/ stress treatments (Chinnusamy & Zhu 2009, Whipple & Holeski 2016). Herrera *et al.,* (2016) also found that epigenome of the nearby individuals were more similar than their genome, especially at small spatial scales. Therefore, population fragmentation of *H. brevisubulatum* might lead to DNA-methylation variation over the last several decades, and such variation increases the evolutionary potential adaptation to the environmental heterogeneity.

Minimal correlation existed between epigenetic and genetic variances in wild barley populations (Figs. 1, 2). Similarly, most researchers agree with that epigenetic variation in natural populations may be independent from genetic one in jatropha (Yi *et al.,* 2010), potato (Marfil *et al.,* 2009), and cotton (Keyte *et al.,* 2006). However, some studies indicated the coordinated genetic-epigenetic adaptive differentiation (Herrera & Bazaga 2010, Paun *et al.,* 2010). Day & Bonduriansky (2011) demonstrated that epigenetic variation might affect the evolution of genetic variation and vice versa, and Ochogavia *et al.,* (2009) showed the strong correlation between methylation and gene expression in *Eragrostis curvula*. Therefore, the relationship between epigenetic and genetic variations is still under debate, and needs further discussion.

In conclusion, the pressures of the HF on the natural populations of *H. brevisubulatum* might result in genomic DNA demethylation, which may re-activate some genes and transposons, and ultimately help plants to adapt to some stress conditions, and lead to epigenetic/genetic variances. The high epigenetic diversity in FPs results in high molecular variance within the populations, consequently, causes higher epigenetic differentiation within populations than that among populations. Thus, epigenetic variance may be more important than genetic one in the response of plants to the HF. The absence of correlation between epigenetic and

geographical distances in *H. brevisubulatum* implies that epigenetic variance may be only specific to habitat environments and fails to reflect the gigantic scale spread history of the species. Even so, this paper primarily studied the relationships between epigenetic variance and the HF, and many questions and hypotheses need to be discussed in wild barley and other organisms.

Acknowledgments

This work was supported by the Science Foundation of Zhejiang Sci-Tech University (ZSTU) (14042008-Y) and National Natural Science Foundation of China (31290211).

References

Avramidou, E.V., I.V. Ganopoulos, A.G. Doulis, A. S. Tsaftaris and F.A. Aravanopoulos. 2015. Beyond population genetics: natural epigenetic variation in wild cherry (*Prunus avium*). *Tree Genet. Genomes*, 11: 1-9.

Chinnusamy, V. and J.K. Zhu. 2009. Epigenetic regulation of stress responses in plants. *Curr. Opin. Plant Biol.,* 12: 133-139.

Day, T. and R. Bonduriansky. 2011. A unified approach to the evolutionary consequences of genetic and nongenetic inheritance. *Am. Nat.,* 178: 18-36.

Earl, D. and B. von Holdt. 2012. Structure harvester: a website and program for visualizing structure output and implementing the Evanno method. *Conserv. Genet. Resour*., 4: 359-361.

Evanno, G., S. Regnaut and J. Goudet. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. *Mol. Ecol*., 14: 2611-2620.

Excoffier, L. and H.E.L. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.,* 10: 564-567.

Falush, D., M. Stephens and J.K. Pritchard. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Mol. Ecol. Notes*, 7: 574-578.

Furrow, R.E. and M.W. Feldman. 2014. Genetic variation and the evolution of epigenetic regulation. *Evolution*, 68: 673-683.

Garcia-Fernandez, A., J.M. Iriondo, A. Escudero, J.F. Aguilar and G.N. Feliner. 2013. Genetic patterns of habitat fragmentation and past climate-change effects in the Mediterranean high-mountain plant *Armeria caespitosa* (*Plumbaginaceae*). *Am. J. Bot.,* 100: 1641-1650.

Guo, W.L., H. Nazim, J. Zahra, R. Wu, J. Wang and B. Liu. 2016. High genetic diversity and structural differentiation in fragmented populations of wild barley (*Hordeum brevisubulatum*). *Pak. J. Bot.,* 48: 2439-2451.

Guo, W.L., R. Wu, Y.F. Zhang, X.M. Liu, H.Y. Wang, L. Gong, Z.H. Zhang and B. Liu. 2007. Tissue culture-induced locus-specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth. et Hook. f. *Plant Cell Rep.,* 26: 1297-1307.

Hashida, S.N., T. Uchiyama, C. Martin, Y. Kishima, Y. Sano and T. Mikami. 2006. The temperature-dependent change in methylation of the *Antirrhinum* transposon *Tam3* is controlled by the activity of its transposase. *Plant Cell*, 18: 104-118.

Herrera, C.M. and P. Bazaga. 2010. Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. *New Phytol.,* 187: 867-876.

Herrera, C.M. and P. Bazaga. 2011. Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. *Mol. Ecol.,* 20: 1675-1688.

Herrera, C.M. and P. Bazaga. 2013. Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (*Aquifoliaceae*) trees. *Bot. J. Linn. Soc*., 171: 441-452.

Herrera, C.M. and P. Bazaga. 2016. Genetic and epigenetic divergence between disturbed and undisturbed subpopulations of a mediterranean shrub: a 20-year field experiment. *Ecol. Evol.,* 11: 3832-3847.

Herrera, C.M., M. Medrano and P. Bazaga. 2016. Comparative spatial genetics and epigenetics of plant populations: heuristic value and a proof of concept. *Mol. Ecol.,* 25: 1653-1664.

Hollander, M. and D. Wolfe. 1973. Nonparametric statistical methods. Wiley, New York.

Huang, F., P. Wang and J. Zhang. 2012. Grasslands changes in the Northern Songnen Plain, China during 1954–2000. *Environ. Monit. Assess.,* 184: 2161-2175.

Kalendar, R., A.J. Flavell, T.H. Ellis, T. Sjakste, C. Moisy and A.H. Schulman. 2011. Analysis of plant diversity with retrotransposon-based molecular markers. *Heredity*, 106: 520-530.

Keyte, A.L., R. Percifield, B. Liu and J.F. Wendel. 2006. Infraspecific DNA methylation polymorphism in cotton (*Gossypium hirsutum* L.). *J. Hered.,* 97: 444-450.

Kim, N.S., M.J. Im and K. Nkongolo. 2016. Determination of dna methylation associated with *Acer rubrum* (red maple) adaptation to metals: analysis of global dna modifications and methylation-sensitive amplified polymorphism. *Ecol. Evol.,* 16: 5749-5760.

Klank, C., J. Ghazoul and A.R. Pluess. 2012. Genetic variation and plant performance in fragmented populations of globeflowers (*Trollius europaeus*) within agricultural landscapes. *Conserv. Genet.,* 13: 873-884.

Kuo, P.C. and L. Zhou. 1980. A preliminary study on the classification and distribution of the genus *Hordeum* L. in China. *Acta. Phytotax Sin.,* 18: 420-427.

Labra, M., A. Ghiani, S. Citterio, S. Sgorbati, F. Sala, C. Vannini, M. Ruffini-Castiglione and M. Bracale. 2002. Analysis of cytosine methylation pattern in response to water deficit in pea root tips. *Plant Biol.,* 4: 694-699.

Lewontin, R.C. 1972. The apportionment of human diversity. *Evol. Biol.,* 6: 381-398.

Li, W.G., W.B. Chen, X.F. Qi, Q.F.Wang and J.M. Chen. 2013. Variation of cytosine methylation in response to water availability in two contrasting growth types of an amphibious plant *Alternanthera philoxeroides*. *Biochem. Syst. Ecol.,* 50: 175-181.

Lira-Medeiros, C.F., C. Parisod, R.A. Fernandes, C.S. Mata, M.A. Cardoso and P.C.G. Ferreira. 2010. Epigenetic variation in mangrove plants occurring in contrasting natural environment. *PLoS One*, 5: e10326.

Manning, K., M. Tor, M. Poole, Y. Hong, A.J. Thompson, G.J. King, J.J. Giovannoni and G.B. Seymour. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat. Genet.,* 38: 948-952.

Marfil, C.F., E.L. Camadro and R.W. Masuelli. 2009. Phenotypic instability and epigenetic variability in a diploid potato of hybrid origin, *Solanum ruiz-lealii*. *BMC Plant Biol.,* 9: 21.

Medrano, M., C.M. Herrera and P. Bazaga. 2014. Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Mol. Ecol.,* 23: 4926-4938.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics,* 89: 583-590.

Ochogavia, A.C., G. Cervigni, J.P. Selva, V.C. Echenique and S.C. Pessino. 2009. Variation in cytosine methylation patterns during ploidy level conversions in *Eragrostis curvula*. *Plant Mol. Biol.,* 70: 17-29.

Pérez-Figueroa, A. 2013. msap: a tool for the statistical analysis of methylation-sensitive amplified polymorphism data. *Mol. Ecol. Resour.,* 13: 522-527.

Paun, O., R.M. Bateman, M.F. Fay, M. Hedrén, L. Civeyrel and M.W. Chase. 2010. Stable epigenetic effects impact adaptation in allopolyploid orchids (*Dactylorhiza*: *Orchidaceae*). *Mol. Biol. Evol.,* 27: 2465-2473.

Preite, V., L.B. Snoek, C. Oplaat, A. Biere, W.H. van der Putten and K.J.F. Verhoeven. 2015. The epigenetic footprint of poleward range-expanding plants in apomictic dandelions. *Mol. Ecol.,* 24: 4406-4418.

Raj, S., K. Bräutigam, E.T. Hamanishi, O. Wilkins, B.R. Thomas, W. Schroeder, S.D. Mansfield, A.L. Plant and M.M. Campbell. 2011. Clone history shapes *Populus* drought responses. *Proc. Nat. Acad. Sci. USA*, 108: 12521-12526.

Rapp, R.A. and J.F. Wendel. 2005. Epigenetics and plant evolution. *New Phytol.,* 168: 81-91.

Reyna-Lopez, G.E., J. Simpson and J. Ruiz-Herrera. 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol. Gen. Genet.,* 253: 703-710.

Rohlf, F. 2000. NTSYS-pc. Numerical taxonomy and multivariate analysis system. Ver. 2.1 Exeter software. Setauket, New York USA.

Salmon, A., J. Clotault, E. Jenczewski, V. Chable and M.J. Manzanares-Dauleux. 2008. *Brassica oleracea* displays a high level of DNA methylation polymorphism. *Plant Sci.,* 174: 61-70.

Schulz, B., R.L. Eckstein and W. Durka. 2014. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Mol. Ecol.,* 23: 3523-3537.

Schulz, B., R.L. Eckstein and W. Durka. 2013. Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. *Mol. Ecol. Resour.,* 13: 642-653.

Shair O.H., E. Askari and P.R., Khan. 2016. Genetic and anatomical analysis of normal and abnormal flowers of date palm cultivar 'Barhy' derived from offshoot and tissue culture. *Pak. J. Bot*., 48: 1061-1065.

Sharma, S., H. Balyan and P. Gupta. 2005. Adaptive methylation pattern of ribosomal DNA in wild barley from Israel Barley. *Barley Genetics Newsletter*, 35: 27-35.

Snell-Rood, E.C., A. Troth and A.P. Moczek. 2013. DNA methylation as a mechanism of nutritional plasticity: Limited support from horned beetles. *J. Exp. Zool. B.,* 320: 22-34.

Tang, X.M., X. Tao, W. Yan, D.W. Ma, D. Li, H. Yang and X.R. Ma. 2014. Analysis of DNA methylation of perennial ryegrass under drought using the methylation-sensitive amplification polymorphism (MSAP) technique. *Mol. Genet. Genomics*, 289: 1075-1084.

Vandegehuchte, M.B. and C.R. Janssen. 2014. Epigenetics in an ecotoxicological context. *Mutat. Res.,* 764-765: 36-45.

Vekemans, X. 2002. AFLP-SURV, Version 1.0. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium.

Verhoeven, K.J., Van P.J. Dijk and A. Biere. 2010. Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. *Mol. Ecol.,* 19: 315-324.

Wang, Z., N. Huang, L. Luo, X. Li, C. Ren, K. Song and J.M.Chen. 2011. Shrinkage and fragmentation of marshes in the West Songnen Plain, China, from 1954 to 2008 and its possible causes. *Int. J. Appl. Earth OBS*., 13: 477-486.

Whipple, A.V. and L.M. Holeski. 2016. Epigenetic inheritance across the landscape. *Front. Genet.*, 7: 189.

Wu, W.Q., M. Yi, X.F. Wang, L.L. Ma, L. Jiang, X.W. Li, H.X. Xiao, M.Z. Sun, L.F. Li and B. Liu. 2013. Genetic and epigenetic differentiation between natural *Betula ermanii* (*Betulaceae*) populations inhabiting contrasting habitats. *Tree Genet. Genomes,* 9: 1321-1328.

Yeh, F., R. Yang, T. Boyle, Z. Ye and J. Mao. 1999. POPGENE version 1.32, the user-friendly shareware for population genetic analysis. molecular biology and biotechnology center, university of alberta, edmonton, AB, Canada (http://www.ualberta.ca/~fyeh/).

Yi C., S. Zhang, X. Liu, H. Bui and Y. Hong. 2010. Does epigenetic polymorphism contribute to phenotypic variances in *Jatropha curcas* L.? *BMC Plant Biol.,* 10: 259.

Zhang X., J. Yazaki, A. Sundaresan, S. Cokus, S.W. Chan, H. Chen, I.R. Henderson, P. Shinn, M. Pellegrini, and S.E. Jacobsen, J.R. Ecker. 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. *Cell*, 126: 1189-1201.

Zhao R, H. Zhang and L. An. 2018. Anthropogenic disturbances affect population size and biomass allocation of two alpine species from the headwater area of the Urumqi river, China. *Pak. J. Bot.,* 50: 199-209.

(Received for publication 11 July 2017)