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HIGH GENETIC DIVERSITY AND STRUCTURAL DIFFERENTIATION IN FRAGMENTED POPULATIONS OF WILD BARLEY (*HORDEUM BREVISUBULATUM*)

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Abstract

Hordeum brevisubulatum(Trin.) Link., a tolerant ofdiverse abiotic stresses,is a wild barley,distributed in Songnen Plain of China. However, many populations of this wild barley are fragmented and/ordispeared during the last few decadesmainly due to anthropogeniceffects. The decrease in fragmented populations may affect the genetic diversity and structure of the populations, and in turn their survival potential.Five fragmented and four unfragmented populations (FP and UFP) in Songnen Plain were analyzed using two types of DNA marker namely, amplified fragment length polymorphism (AFLP) and sequence-specific amplified polymorphism (SSAP). The genetic diversities (0.162_{AFLP} and 0.239_{SSAP}) of five FPs [SB1, SB2, DBS, QZJ and QG]were higher than those (0.126_{AFLP} and 0.20_{SSAP}) of four UFPs [SS1, SS2, FY1 and FY2], although the later one (the UFPs) had large population size. Moreover, the 5FPsalso showed higher population genetic differentiation (G_{ST}, 0.197_{AFLP} and 0.192_{SSAP}) comparing with 4 UFPs (0.086_{AFLP} and 0.108_{SSAP}). The habitat fragmentation could improve the genetic variability, and lead to heterogeneous impact on different genome regions in *H. brevisubulatum*.

Key words: *Hordeum brevisubulatum*,Habitat fragmentation,Genetic diversity,Population structure, AFLP, SSAP.

Introduction

The increasing anthropogenic impactin the past decades has transformed vast natural habitats into different types of landscape, such as farmlands, roads, mines and urban areas (Aguilar *et al*., 2008), consequently, large continuous habitats are separated into small and isolated patches. Plants in fragmentized habitats may therefore experience reduced population size and low genetic diversity in natural species (Young *et al*.,1996, Mcgarigal & Cushman 2002, Fahrig 2003, Lowe *et al*., 2005, Culley *et al*., 2007, Quinteros-Casaverde *et al*., 2012, McKechnie & Sargent 2013). For instance, Hundera *et al*., (2013)had demonstrated that the species richness and individual densities of epiphytic orchid were severely reduced in the managed coffee and natural fragmented forestsin comparison with those of natural continuous forest. Moreover, small populations of *Urosaurus nigricaudus* located in isolated patches tended to go extinct more frequently than larger ones (Munguia-Vega *et al*., 2013). In addition, similar genetic effects of habitat fragmentation on natural species had also been investigated in several other types of landscape, such as grasslands (Young *et al*., 1996), woodlands (Jump & Penuelas 2006,Kolb & Durka 2013), agricultural areas (Culley & Grubb 2003), and urban areas (Culley *et al*., 2007). In contrast, however, the habitat fragmentations had only slightly effects on the genetic diversity of fragmented populations relative to these of continuously distributed populations of woody plants (Young *et al*., 1996), perennial herbs(Young *et al*., 1999, Pluess & Stöcklin 2004, Kuss *et al*., 2008, Klank *et al*., 2012, Vandepitte *et al*., 2013). These findings indicated that the influences of habitat fragmentation on the population genetic variability were complex and further investigations were required to be addressed for different ecological systems with numerous plant species.

Grassland is a fragile ecosystem seriously threatened by human activities and global climate change. Songnen Plain in Northeast China is one of the most significantly altered biological hotspots on Earth (Huang *et al*., 2012), located in the eastern edge of Eurasia Steppe with saline-alkaline soil and saline-alkaline lakes, developed from quaternary stratigraphy, a rift basin, and surrounded by mountains at three sides (Luo *et al*., 2003). Surprisingly, the Plain has been disturbed roughly by human activities since mid of the 20th century (Wang *et al*., 2008, 2011, Huang *et al*., 2012). Consequently, lots of natural continuous habitats in Songnen Plain had been transformed into agricultural and urban areas. For example, marshes and grasslands in this area had decreased by 74% and 54%, and cropland and saline wasteland expanded 22% and 612% (Wang *et al*., 2011), and the numbers of grassland patch increased by 1,378, while the patch size of grasslands declined (Huang *et al*., 2012). These attributes, therefore, suggested that Songnen Plain could be the ideal ecosystem to be addressedfor evaluating the genetic variability of natural species by the habitat fragmentation. However, few reports showed the trends of the grassland fragmentation and disturbance on geo- and bio-changes in Songnen Plain (Han *et al*., 2009, Wang *et al*., 2009), the influences of habitat fragmentation on the genetic variability of natural species in this area was still unknown(Han *et al*., 2009).

Hordeum brevisubulatum (Trin.) Link., one of the wild perennial and outcrossing autotetraploid species of genus *Hordeum* (Guo & Zhou 1980), is the dominant species at some undisturbed places or dispersed around saline-alkaline patch, while scattered in fragmented habitats in Songnen Plain (Guo *et al*., 1998). Wild barley is a broad-spectrum tolerant species to diverse abiotic stresses, including drought, salinity and alkalinity (Guo *et al*., 1998), and thus has the survival ability to different environmental stress conditions, in particular to the saline-alkaline patches. Here, four unfragmented (UFP)

and five fragmented populations (FP) of wild barley species were selected to study their genetic diversity and differentiation, and the influence of habitat fragmentation on wild barley population genetic variation.

In our previous studies, we reported the genetic and epigenetic diversities of *H. brevisubulatum*with limited populations (Li *et al*., 2008). In this study, we employed amplified fragment length polymorphism (AFLP) and sequence-specific amplified polymorphism (SSAP) to evaluate the genetic consequences of habitat fragmentation on wild barley with large samples. AFLP was developed by Vos *et al*., (1995) and widely applied in genetic studies due to its capability to detect genetic polymorphisms in different genomic regions simultaneously without any need for prior sequence information (Wu *et al*., 2013). Retrotransposon *BARE-1*, the first intact retrotransposon cloned from *H. vulgare* (Manninen & Schulman 1993) with high-copy number ($1.588 \times 10^4 \pm 0.085 \times 10^4$) and $2.145 \times 10^5 \pm 0.012 \times 10^5$ LTRs (Long-terminal repeats), accounted for 9.6% of the total*Hordeum* genome (Kalendar *et al*., 1999, Soleimani *et al*., 2006), activated in recent time (Vicients *et al*., 2001). Thus, *BARE-1* had been employed to evaluate the polymorphisms within or near its sequences in diverse *Hordium* species by SSAP (Bonchev & Parisod 2013, Kalendar *et al*., 2000, Konovalov *et al*., 2010, Kalendar *et al*., 2011, Schulman *et al*., 2012, Waugh *et al*., 1997), and its variations may have some correlation with its micro-habitats (Kalendar *et al*., 2000). Therefore, the aims of this study were focused on the following questions: (1) to evaluate the population genetic diversity, differentiation and structure of the nine natural populations (4 UFPs and 5 UFPs) of wild barley selected from Songnen Plain; (2) to address the influences of habitat fragmentation on the population genetic variability and structure; (3) to test genetic variability/heterogeneity among different genomic regions.

Materials and Methods

Population sampling and DNA extraction: A total of 235 individuals (19-38 individuals per population) from nine populations of *H. brevisubulatum* were sampled from the Songnen Plain (Fig. 1b), of which four (SS1, SS2, FY1 and FY2) and five (SB1, SB2, QG, DBS and KZJ) populations were sampled from unfragmented and fragmented habitats, respectively. A fragmented population here was defined as wild barley distributing within a discrete location or parted by long distance, often isolated by agricultural field, road, lake or thorpe, and most sites were separated at least 1.8 kilometer (two populations were isolated by a town with a width of 1.8 km). The detail of the populations is referred to Table 1. The geographical distances between populations were computed using the formula of the great-circle distance between loci (http://www.movable-type.co.uk/scripts/latlong.html, Table 1), and formed a geographical distance matrix (Table 2). Seeds of *H. brevisubulatum* individuals were collected along a curve transected with a minimum spacing of at least 20m between sampled loci to reduce the likelihood among the collected individuals. Seeds from each plant were then germinated in glasshouse and 15-day-old single plant was also randomly selected from those seedlings for further analysis. Genomic DNAs were extracted from the totally expanded leaves by using a modified CTAB method (Kidwell & Osborn 1992) and purified by phenol extractions. The quality and quantity of extracted DNA were studied using gel electrophoresis and spectrometric assays.

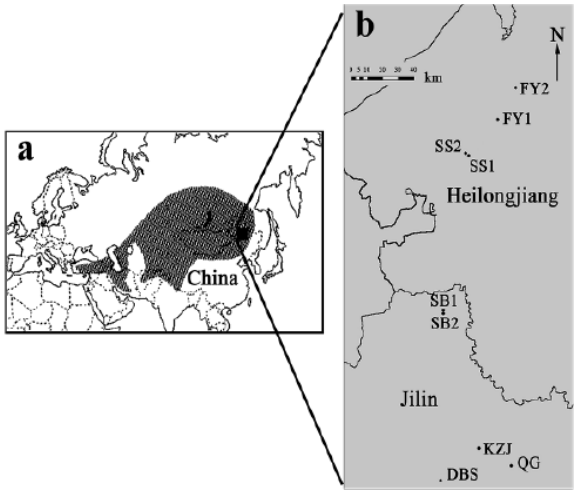

Fig. 1.Geographic distribution of *Hordeum brevisubulatum* in the world (a) (Von Bothmer *et al*., 1995), and the sampling loci (b) zoomed from black box in (a).

AFLP and SSAP amplifications: AFLP amplification followed the protocol described by Vos *et al*., (1995) with minor modifications (Liu *et al*., 2001). In brief, total genomic DNA (500 ng) was digested with *Eco*RI (20 U/μL, NEB, England) and *Mse*I (10 U/μL, NEB, England). *Eco*RI and *Mse*I adaptors were then ligated to the digested DNA fragments by using T4-ligase (Takara, Dalian, China), and pre-amplification was conducted using a combination of *Eco*RI and *Mse*I primers. Each PCR reaction contained 0.3 μM [*Mse*I + 1], 0.3 μM [*EcoR*I (or *Pst*I) + 1], 1.0 U of rTaq DNA polymerase (Takara, Dalian, China), 0.2 mM of each dNTP, and 2 μL of diluted restriction–ligation sample, in 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 30 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 [*EcoR*I + 3] primers, 0.15 μM [*Mse*I + 3 primer], 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 min, 56°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 30 min. The amplifications in this study were performed in a PTC-100 Thermal Cycler (The MJ Research Inc., Waltham, MA).

Table 1. Summary of the sampled loci and population genetic diversities of *Hordeum brevisubulatum*.

Population name		Location	Coordinates	HS (m*m) ID (plants/m^2)	SN	Habitat	AFLP		SSAP	
							PPL (%)	H_e (SD)	PPL (%)	H_e (SD)
FP	SB1	Zhenlai, JL	123°42.270′ 46°06.463′	1500*200 (~3)	26.0	Surrounded by Road, field and town	34.0	0.175 -0.006	92.8	0.246 -0.009
	SB2	Zhenlai, JL	123°42.431′ 46°05.476′	1000*100 (~3)	35.0	Surrounded by Road, field and town	29.3	0.161 -0.006	96.1	0.257 -0.008
	QG	Qianguo, JL	124°26.096′ 44°57.812′	10000*10000 (~1)	23.0	Surrounded by field	36.8	0.185 -0.007	51.2	0.233 -0.009
	DBS	Qianan, JL	123°39.726′	1500*1500	38.0	Surrounded by field and a river	32.2	0.158	49.6	0.251
	KZJ	Qianan, JL	124°05.077′ 45°05.693′	800*300 (~0.1)	19.0	Surrounded by field and a lake	22.2	0.130 -0.006	38	0.210 -0.009
	Average				28.2		30.9	0.162	65.5	0.239
UFP	SS1	Qiqihaer, HLJ	124°00.610′ 47°14.782′	10000*10000 (~8)	28.0	Grassland	24.8	0.129 -0.006	41.1	0.209 -0.009
	SS2	Qiqihaer, HLJ	123°58.236′ 47°15.897′	10000*10000 (~8)	23.0	Grassland	25.5	0.132 -0.006	41.9	0.215 -0.009
	FY1	Fuyu, HLJ	124°33.595′ 47°44.032′	40000*40000 (~14)	21.0	Grassland	23.2	0.126 -0.006	34.6	0.190 -0.009
	FY2	Fuyu, HLJ	124°20.725′ 47°30.425′	40000*40000 (~14)	22.0	Grassland	21.5	0.115 -0.006	35.7	0.187 -0.008
	Average				23.5		23.8	0.126	38.3	0.200
Total		**9**			**235.0**		**27.7**	**0.146**	**53.4**	**0.222**

JL: Jilin province, HLJ: Heilongjiang province, Coordinates: Longitude (N)-latitude (E), HS: Habitat size (m*m), ID: Individual density, SN: Number of sampling plants, PPL: Percentage of polymorphic loci, FP: Fragmented population, UFP: Unfragmented population, He: Unbiased expected heterozygosity, SD: Standard deviation.

Table 2. Matrices of pairwise F_{ST} and geographical distance among *Hordeum brevisubulatum* populations.

		FP				UFP				
		SB1	**SB2**	**QG**	**DBS**	**KZJ**	**SS1**	**SS2**	**FY1**	**FY2**
						F_{ST}				
FP	SB1	0	0.068	0.124	0.172	0.224	0.282	0.275	0.283	0.296
	SB2	0.104	0	0.074	0.099	0.149	0.224	0.221	0.23	0.238
	QG	0.073	0.121	0	0.131	0.211	0.278	0.272	0.281	0.289
	DBS	0.15	0.068	0.136	0	0.104	0.205	0.208	0.214	0.212
	KZJ	0.224	0.178	0.189	0.119	0	0.252	0.242	0.245	0.257
UFP	SS1	0.298	0.263	0.267	0.232	0.253	0	0.008	0.047	0.051
	SS2	0.289	0.257	0.258	0.226	0.251	0.017	0	0.021	0.029
	FY1	0.316	0.282	0.277	0.244	0.287	0.057	0.06	0	0.013
	FY2	0.336	0.302	0.29	0.261	0.301	0.084	0.069	0.023	0
		Geographical distance between populations (Kilometer)								
FP	SB1	0.000								
	SB2	1.845	0.000							
	QG	140.185	138.033	0.000						
	DBS	62.203	138.120	136.339	0.000					
	KZJ	31.601	116.934	115.686	42.460	0.000				
UFP	SS1	256.287	128.695	129.761	268.646	241.167	0.000			
	SS2	259.216	130.679	134.062	268.707	244.165	3.615	0.000		
	FY1	283.402	173.214	165.053	298.551	270.522	39.935	40.246	0.000	
	FY2	308.376	202.565	194.738	327.189	295.644	69.521	69.408	30.575	0.000

AFLP: Up diagonal, SSAP: down diagonal. FP: Fragmented population, UFP: Unfragmented population

SSAP amplification was performed according to the method (Waugh *et al*., 1997) with a little modification and referred to AFLP. *Eco*RI was replaced by *Pst*I in AFLP (10U/µl; NEB, England). The markedly difference between SSAP and AFLP was that one selected primer (*Pst*I or *Mse*I) combined with a specific primer designed from retrotransposon *Bare-1* (Waugh *et al*., 1997) in SSAP to produce polymorphisms. All the adaptors, pre-primers and selected primers used in AFLP and SSAP were referred to Table 3.

A total of 30 and 90 selected primer pairs were screened for SSAP and AFLP, respectively, using silver stained sequencing gel (Li *et al*., 2008). All the primer pairs of the AFLP and SSAP were initially screened using three DNA samples randomly selected from 235 wild barley plants with 3 repeats separately, and primer pairs that produced clear and reproducible bands were selected for subsequent analyses. Then, two independent amplifications using the selected primer pairs (Table 4) on all 235 individuals were carried out and only the clear and reproducible bands ranging from 100 bp to 500 bp were scored.

Analyses of AFLP and SSAP data: The scored bands of AFLP and SSAP were transformed into binary tables, wherein "1" and "0" were assigned for the presence and absence of a band at a particular locus, respectively. The number of loci (NL), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL) and polymorphic information content (PIC), PIC= $2f^*(1-f)$, where f is the percentage of the polymorphic loci out of the total loci, were calculated for each selected primer pair (Anderson *et al*., 1993).

The matrices for the presence/absence of AFLP and SSAP fragments were used to calculate the allele frequencies by aBayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). The PPL (5% level), genetic diversity (H_e,Nei 1973) and pair-wise population genetic differentiation (F_{ST}, 1000 random permutations) were calculated according to Lynch and Milligan (1994) using AFLP-SURV version 1.0 (Vekemans 2002). In addition, G_{ST}(the coefficient of gene differentiation, G_{ST}= (H_T-H_S)/H_T (H_T: total genetic diversity, i.e., expected heterozygosity, H_S: genetic diversity within population), Nei 1987) was calculated using POPGENE version 1.32 (Yeh *et al*., 1999). Mendelian segregation and Hardy-Weinberg Equilibrium (HWE) within populations were assumed. Individual-based neighbor joining (NJ) (Saitou & Nei 1987) trees were generated using PAUP 4.0 (Swofford 2002). In addition, the population-based NJ trees were constructed using *neighbor* program in PHYLIP 3.65 (Felsenstein 1986). Principle coordinate analysis (PCOA) based on Jaccard's similarity index (Jaccard 1908) using NTSYS-pc version 2.10e (Rohlf 2000). The correlations between the indexes of Nei's genetic diversities (H_e), mean percentage of polymorphic loci (PPL), Isolation By Distance (IBD) and Individual

Density (ID) were determined by Spearman's non-parametric correlations(Hollander & Wolfe 1973) 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. Population genetic differentiation was estimated by analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992)based on matrices of squared standard Euclidean distances between all pairs of those two polymorphic data using the software Arlequin, version 3.5 (Excoffier *et al*., 2005). The Bayesian-based software STRUCTURE (Falush *et al*., 2007) was employed to investigate the genetic structure of the nine populations with the LOCPRIOR model (Hubisz *et al*., 2009). The number of genetic clusters K was performed from 1 to 9 using a non-admixture model with independent allele frequencies between populations. The burn-in period is 50,000 with 500,000 MCMC (Markov Chain Monte Carlo) replications. Delta K (the true number of genetic groups, Evanno *et al*., 2005) was estimated using an *ad hoc* quantity by STRUCTURE HARVESTER (Earl & vonHoldt 2012). CLUMMP, version 1.1.1 (Jakobsson & Rosenberg 2007) was used with the greedy algorithe and 10,000 random input orders of 9 independent structure runs to determine the optimal alignment of clusters across individual runs for selected Ks, and *Distruct*, version 1.1 (Rosenberg 2004) was employed to create nice plots from CLUMPP results.

Results

Characteristics of primer pairs: 717 loci were obtained from 235 individuals amplified by 10 AFLP selective primer pairs (Table 4). The PPL of each primer pair varies from 37% with *Eco*RI+AGG-3′/*Mse*I+CTT-3′ to 72% with *Eco*RI+ACC-3′/*Mse*I+CCA-3′, with an average of 53%. Moreover,combination of *Eco*RI+ACC-3′/*Mse*I+CCA-3′ resulted in the highest PIC (0.210) index, whereas the primer pair of *Eco*RI+AGA-3′/*Mse*I+CCA-3′ produced the lowest PIC (0.063) index. For SSAP, 387 loci were produced from 12 selective primer pairs (Table 4). The PPL of each primer pair ranged from 48% (*Mse*I+CCA-3′, the lowest PIC=0.089) to 97% (*Pst*I+ATG-3′, the highest PIC=0.293) with an average of 73%. Although SSAP primer pair produced fewer bands (32.25 loci per primer pair) than that of AFLP (71.7 loci per primer pair), higher genetic diversity (average PIC = 0.194) was discovered by SSAP than that by AFLP (average PIC = 0.120). These results indicated that DNA sequences within and near retrotransperson *BARE-1*might have more variation than whole genome in wild barley.

Table 3.Adaptors and primers used in this study.

Type/Code	Sequences
Adapters	
*Mse*I-adapter I	5'-GACGATGAGTCCTGAG-3'
*Mse*I-adapter II	5'-TACTCAGGACTCAT-3'
*Eco*RI-adapter I	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adapter II	5'-AATTGGTACGCAGTC-3'
*Pst*I-adapter I	5'-CTCGTAGACTGCGTACATGCA-3'
*Pst*I-adapter II	5'-TGTACGCAGTCTAC-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'
*Mse*I+C	5'-GATGAGTCCTGAGTAAC-3'
*Pst*I+0	5'-GACTGCGTACATGCAG-3'
*Hpa*II/*Msp*I+0	5′-ATCATGAGTCCTGCTCGG-3′
Selective primer combinations used in AFLP	
	*Eco*RI+**AAG**-3′/*Mse*I+**CTA**-3′ *Eco*RI+**ACA**-3′/*Mse*I+**CTC**-3′
	*Eco*RI+**ACA**-3′/*Mse*I+**CTT**-3′ *Eco*RI+**ATC**-3′/*Mse*I+**CTT**-3′
	*Eco*RI+**AGA**-3′/*Mse*I+**CCA**-3′ *Eco*RI+**ATC**-3′/*Mse*I+**CAG**-3′
	*Eco*RI+**ACA**-3′/*Mse*I+**CTA**-3′ *Eco*RI+**ACT**-3′/*Mse*I+**CTC**-3′
	*Eco*RI+**AGG**-3′/*Mse*I+**CTT**-3′ *Eco*RI+**ACC**-3′/*Mse*I+**CCA**-3′
Selective primers used in SSAP	
Bare-1 primer	5′-CTAGGGCATAATTCCAACAA-3′ (Waugh *et al*., 1997)
	*Mse*I+**CAC**-3′ *Mse*I+**CTA**-3′ *Mse*I+**CCA**-3′
	*Mse*I+**CTT**-3′ *Mse*I+**CAT**-3′ *Pst*I+**ATC**-3′
	*Pst*I+**AGA**-3′ *Pst*I+**CCA**-3′ *Pst*I+**ATG**-3′
	*Pst*I+**ACT**-3′ *Pst*I+**CTG**-3′ *Pst*I+**CCG**-3′

Table 4.The characteristics of primers used in AFLP and SSAP.

Marker type/Primer combination	NL	NPL	PPL(%)	PIC
AFLP				
*Eco*RI+**ATC**-3′/*Mse*I+**CAG**-3′	80	45	56	0.129
*Eco*RI+**ACA**-3′/*Mse*I+**CTA**-3′	78	36	46	0.109
*Eco*RI+**ACT**-3′/*Mse*I+**CTC**-3′	63	36	57	0.136
*Eco*RI+**AGG**-3′/*Mse*I+**CTT**-3′	59	22	37	0.083
*Eco*RI+**ACC**-3′/*Mse*I+**CCA**-3′	64	46	72	0.21
*EcoR*I+**AAG**-3′/*Mse*I+**CTA**-3′	72	37	51	0.097
*Eco*RI+**ACA**-3′/*Mse*I+**CTC**-3′	71	44	62	0.153
*Eco*RI+**ACA**-3′/*Mse*I+**CTT**-3′	70	31	44	0.096
*Eco*RI+**ATC**-3′/*Mse*I+**CTT**-3′	72	39	54	0.12
*Eco*RI+**AGA**-3′/*Mse*I+**CCA**-3′	88	44	50	0.063
Total	717	380		
Average	71.7	38	53	0.12
SSAP				
Bare-1 primer/*Mse*I+**CAC**-3′	39	31	79	0.197
Bare-1 primer/ *Mse*I+**CAT**-3′	32	19	59	0.159
Bare-1 primer/ *Mse*I+**CTA**-3′	31	21	68	0.145
Bare-1 primer/ *Mse*I+**CTT**-3′	42	26	62	0.159
Bare-1 primer/ *Mse*I+**CCA**-3′	42	20	48	0.089
Bare-1 primer/ *Pst*I+**ATG**-3′	32	31	97	0.293
Bare-1 primer/ *Pst*I+**ATC**-3′	29	26	90	0.266
Bare-1 primer/ *Pst*I+**ACT**-3′	27	22	81	0.204
Bare-1 primer/ *Pst*I+**AGA**-3′	31	26	84	0.226
Bare-1 primer/ *Pst*I+**CTG**-3′	28	15	54	0.136
Bare-1 primer/ *Pst*I+**CCA**-3′	29	25	86	0.235
Bare-1 primer/ *Pst*I+**CCG**-3′	25	21	84	0.221
Total	387	283		
Average	32.25	23.58	73	0.194

NL: number of loci, NPL: number of polymorphic loci, PPL: percent of polymorphic loci, and PIC: polymorphic information content

Genetic diversity of fragmented and unfragmented populations: The percentage of polymorphic loci (PPL) at species level showed that 27.7% and 53.4% loci for AFLP and SSAP, respectively. For each population, the PPL varied from 21.5% (FY2) to 36.8% (SB2) for AFLP and from 34.6% (FY1) to 96.1% (QG) for SSAP (Table 1). Similarly, the H_e for each population ranged from 0.115 (FY1) to 0.185 (SB2) in AFLP and 0.187 (FY1) to 0.257 (QG) in SSAP. At the species level, the H_e was 0.146 for AFLP and 0.222 for SSAP (Table 1). Furthermore, genetic diversity in populations of the FPs showed significantly (p_{He}< 0.01)higher than those of the UFPs with both AFLP and SSAP (Table 1), and the correlations between genetic diversities (H_e) and individual densities (ID, Table 1) were significantly ($p<0.01$) negative($r_{AFLP\ (ID\text{-}He)} = -0.742$, $p<0.01$ and $r_{SSAP\ (ID\text{-}He)} = -0.795$, $p<0.01$, Spearman's nonparametric correlation), indicating that lower density of wild barley plants in a population blocked the genetic communication among the individuals. Accordingly, low level of rare allele (allele frequency < 10% at the population level) was observed in the UFPs (15 alleles per population) than the FPs (23.4 alleles per population) by the SSAP (Fig. 2a). However, the UFPs showed relatively high level of rare allele (47 alleles per population) than the FPs (32.8 alleles per population) by AFLP (Fig. 2b). The rude IBD (Isolation by distance) analyses also revealed that significant positive correlations between genetic and geographical distances within the UFPs ($r=0.81$ and $r=0.95$ of AFLP and SSAP respectively, $p<0.05$, Fig. 3. c,d), no correlations in the FPs($r = 0.10$ and $r = -0.10$ of AFLP and SSAP respectively,$p>0.05$, Fig. 3a,b). Moreover, the correlation between genetic and geographical distances was partly consistent to IBD model(Wright 1943) in total 9 wild barley populations($r = 0.73$ using *Fst* distances of AFLP and SSAP, Table 2), suggesting that genetic communication barriers severelyexisted among the FPs in wild barley.

In addition, those indices indicated that AFLP had similar patterns of genetic variations with SSAP in detecting population in wild barleyas the correlation (r_{He} =0.850, $p<0.01$) between AFLP and SSAP confirmed the hypothesis.

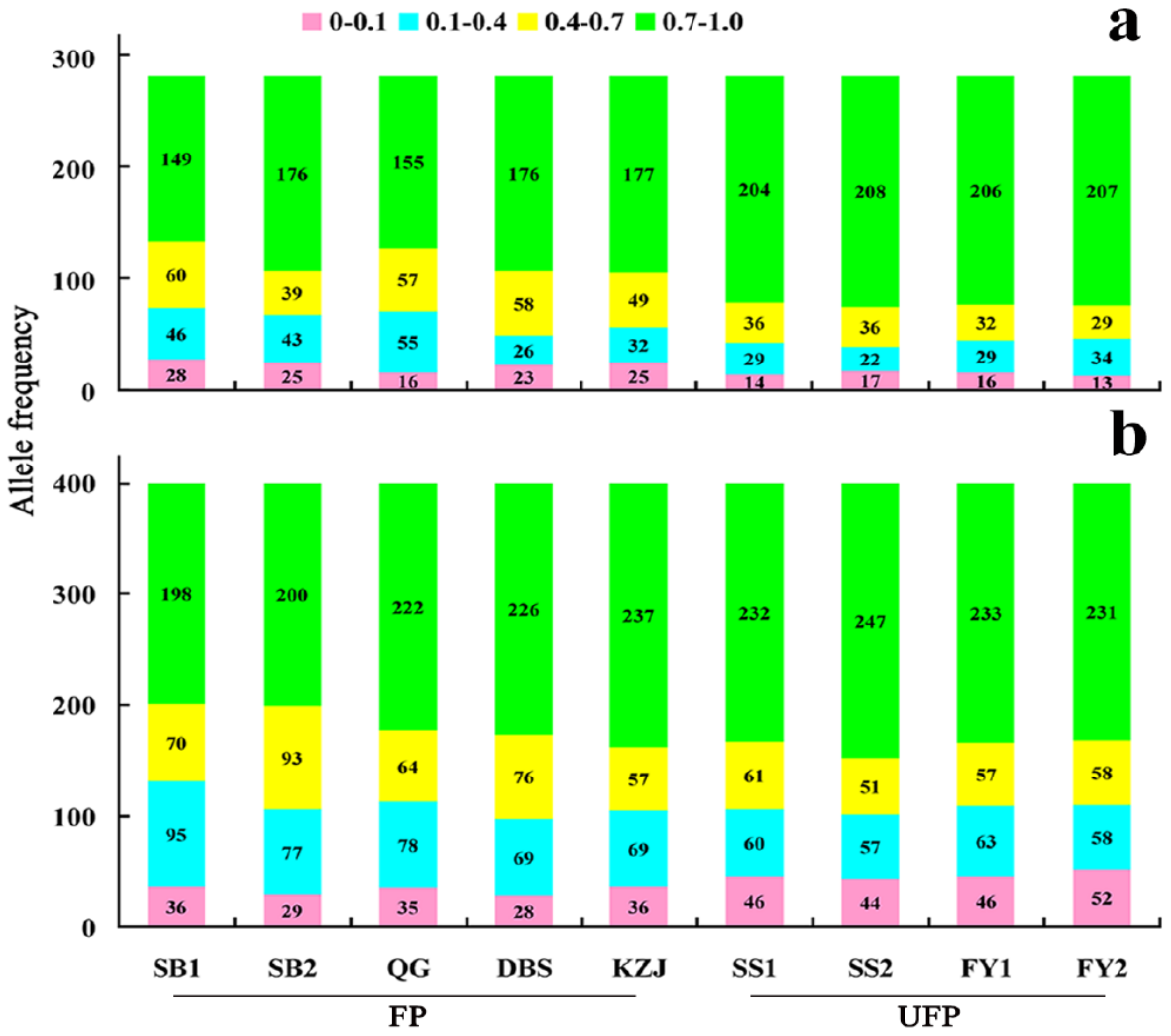

Fig. 2.Histograms of allele frequency of nine *Hordeum brevisubulatum* populations based on SSAP (a) and AFLP (b). FP: Fragmented population, UFP: Unfragmented population.

Genetic differentiation of fragmented and unfragmented populations: Averages of genetic differentiations based on SSAP (G_{ST} = 0.291, F_{ST} = 0.199 (SD = 0.093)) were slightly higher than those based on AFLP (G_{ST} = 0.277, F_{ST} = 0.181 (SD = 0.096)). Furthermore, pairwise population genetic differentiation (F_{ST}, table 2) demonstrated that FP group showed significantly higher population genetic differentiation (Average of $F_{ST\text{-}AFLP}$ = 0.1356 (SD = 0.054) and $F_{ST\text{-}SSAP}$ = 0.1362 (SD = 0.050)) than those of UFPgroup (Average of $F_{ST\text{-}AFLP}$ = 0.0282 (SD = 0.018) and $F_{ST\text{-}SSAP}$ = 0.0517 (SD = 0.0263)) in both AFLP (t-test, p<0.01) and SSAP (t-test, p<0.05).Notably, the indices of pairwise differentiation (F_{ST}) between FPs and UFPs were significantly higher than those between populations within FP and UFP groups (Table 2, p<0.01 for both AFLP and SSAP datasets). This also proved bythe AMOVA analysis (Table5), revealing that variations among populations within a group (10.86% for AFLP and 11.38% for SSAP) were obviously lower than variations between the groups (21.40% for AFLP and 22.23% for SSAP). Moreover, the division of individuals from FP and UFP groups in two distinct clusters was also confirmed by PCOA (Fig. 4), and by STRUCTURE analyses with the delta K equals to 2 in both AFLP and SSAP (Fig. 5)

To address the factors underlying the spatial genetic structure of the nine populations, NJ trees were reconstructed at both individual and population levels (Fig. 6). The population phylogenetic trees based on AFLP (Fig. 6c) and SSAP (Fig. 6d) dataset showed that the nine populations were separated into two clades, similar with the results of PCOA (Fig. 4) and STRUCTURE (Fig. 5). The similar results were also observed in the individual-based phylogenetic trees (Fig. 6a, b) that all the individuals from the same group were clustered together as a monophyletic clade. These attributes indicated that the four populations within UFP group might have diverged recently. However, there were more ramifications in FP group (SB1, SB2, QG, KZJ, and DBS) comparing to UFP group (Fig. 6a, b). These five FPs were clustered into two subclades based on AFLP (Fig. 6a), and some plants belonging to different populations were grouped together (Fig. 6a, b), similar with the results that k = 3 and k = 5 in STRUCTURE analysis (Fig. 5).

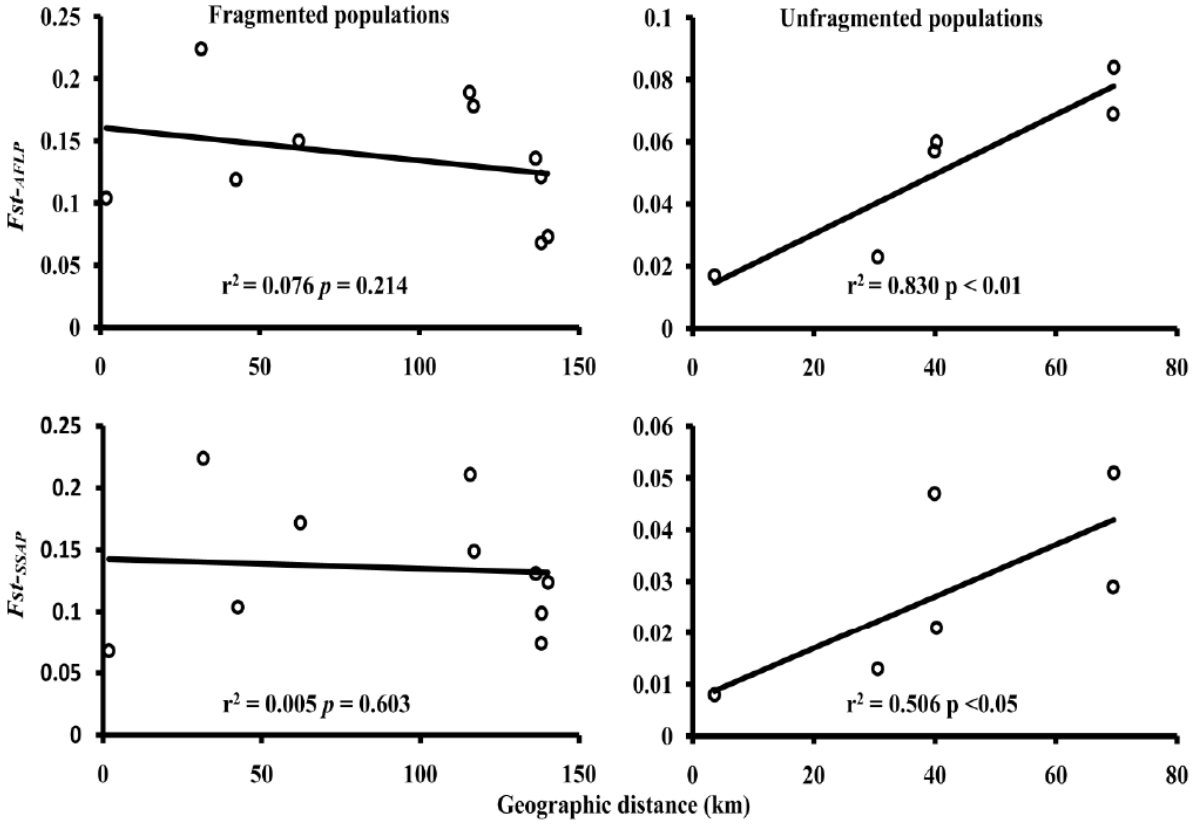

Fig. 3. The correlations between pairwise *Fst* and pairwise geographic distance among *Hordeum brevisubulatum* populations within fragmented and unfragmented groups.

Discussion

Generally, habitat fragmentation could result in the loss of genetic diversity (Willi *et al*., 2006, Quinteros-Casaverde *et al*., 2012, McKechnie & Sargent 2013). However, based on AFLP and SSAP datasets, higher genetic diversities were discoveredin the FPs (Table 1) of wild barely. Various factors might have contributed to the high genetic diversity of the FPs. For instance, the different demographic background of the nine populations that led to the separation of FP and UFP groups into two distinct genetic clusters (Figs. 4, 5 and 6), indicated that populations within both the groups might have different evolutionary process. Although habitat fragmentation could have been happened within 50 year span (Wang *et al*., 2008, 2011, Huang *et al*., 2012), populations within FP group might possess relatively higher ancestral genetic polymorphisms than those of UFP group, but still kept unknown. The genetic diversity could be affected by life history traits of natural species (Hamrick & Godt 1996), relatively higher genetic diversity might be expected in long-lived perennials with an outcrossing breeding system than annual selfing species (Hamrick & Godt 1996, Kreivi *et al*., 2005). In this point, the perennial and out crossing traits of *H. brevisubulatum* might cushion the influences of habitat fragmentation on the loss of genetic diversity(Guo & Zhou 1980). Furthermore, relatively high tolerance to loss of genetic diversity in polyploid species (Moody *et al*., 1993, Perez-Collazos & Catalan 2006, Klank *et al*., 2012)could be reflected in terms of high genetic diversity in the FPs of*H. brevisubulatum*. On the other hand, the Songnen Plain has only been severely fragmentized in the last five decades (Huang *et al*., 2012), and population genetic diversity in wild barley may take more generations to reach equilibrium. Despite high genetic diversities observed in the FPs, the proportion of rare alleles based on AFLP in the UFPs were obviously greater than that of the FPs (Fig. 2b), suggesting the loss of rare alleles tendency in the FPs. In the past, loss of rare alleles hadbeen observed in *Rutodosis leptorrhynchoides* (Young *et al*., 1999), *Brongniartia vazquezii* (González-Astorga & Núñez-Farfán, 2001), *Berchemiella wilsonii* (Kang *et al*., 2005), and *Andean Polylepis* (Gareca *et al*., 2013). These also implied that the UFPs unlikely resulted from recent colonization or bottleneck effects based on the higher number of rare alleles in UFPs (Fig. 2b), although the UFPs had lower genetic diversities. Recent colonization or bottleneck effects may lead to losing of rare alleles due to random genetic drift increase (Hyten *et al*., 2006, Meyer & Purugganan 2013). Conclusively, our findings confirmed that habitat fragmentation did affect thegenetic variability in the 5 natural populations of wild barley.

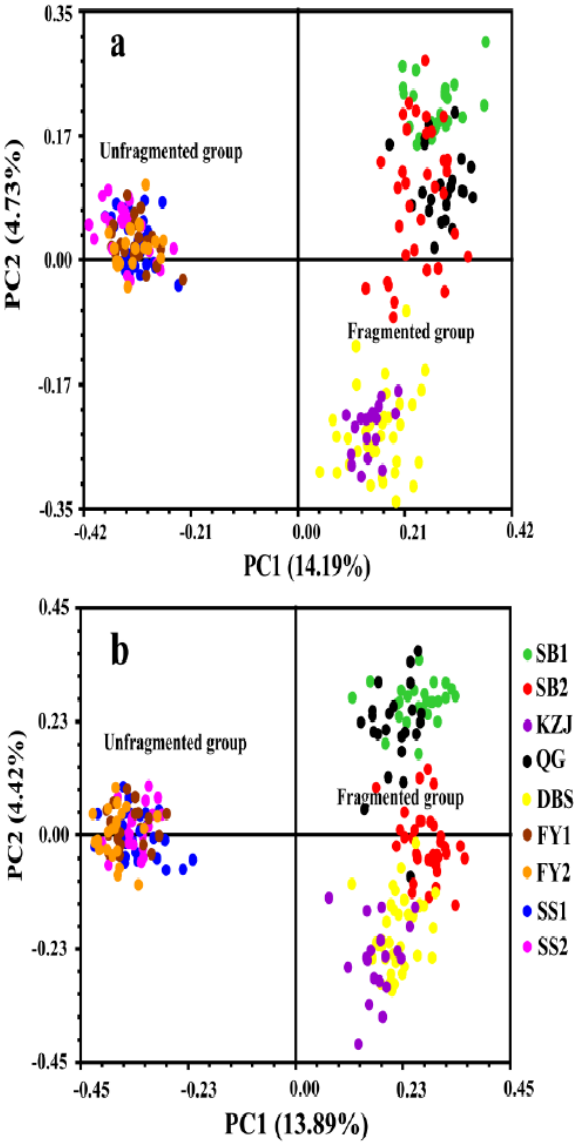

Fig. 4. Principle coordinates analysis (PCoA) using Jaccard's similarity matrixes of 235 *Hordeum brevisubulatum* individuals. (a): AFLP, (b): SSAP.

Our findings also confirmed that FP usually showed high genetic differentiation due to the random genetic drift within populations and deceasing of genetic communication between populations (Young *et al*., 1996, Tallmon *et al*., 2002, Culley *et al*., 2007, Honnay *et al*., 2007, Pereira *et al*., 2010, Michalski & Durka 2012, Huang *et al*., 2012, Kolb & Durka 2013). The high population genetic differentiation might possibly arose due to habitat fragmentation, which could cause the occurrence of genetic communication barriers between isolated populations. This inference was confirmed by the IBD analyses results (Fig. 3), non-significant correlations among the FPs, whereas, significantly positive correlations were observed between genetic and geographical distances within the UFPs. In addition, soil stresses by salitiny or alkalinity resulted from anthraopogenic activities in most fragmented habitats in Songnen plain (Huang *et al*., 2012) might be one of the possible causes for increasing genetic differentiation. The habitat with drought stress in which the variation of the retrotransposon *BARE-1*was correlatedto its micro-habitatswith high genetic diversity and differentiation (Kalendar *et al*., 2000). We also observed the similar results, and more rare alleles(23.4 alleles per population) were detected in the FPs using SSAP (Fig. 2a). Thus, habitat fragmentation in wild barley with stress conditions may affect its population spatial structure in Songnen Plain, but the influence correlation between habitat fragmentation and stresses on population variation should be studied further.

Although AFLP and SSAP both showed the same trend (r_{He} =0.850, $p<0.01$)for genetic diversity and structure of wild barley populations affected by fragmented habitats, but comparatively higher genetic diversity anddifferentiation were observed in SSAP than AFLP for each population (Table 1). SSAP showed more polymorphism than AFLP in *Pisum*, *Hordeum*, *Citrus*, *Malus*, *Oryza,* and *Aegilops* (Kalendar *et al*., 2011), for example, SSAP based on *Bare-1* has 25% of polymorphic ratio higher than AFLP in *H. vulgare* (Waugh *et al*., 1997), and also similar with our results.Moreover, two times more polymorphic loci (P) in barley had been reported with SSAP markers than AFLP (Waugh *et al*., 1997, Kalendar *et al*., 2011) and the distribution of large number of copies of *BARE-1* throughout the genome of wild barley (Soleimani *et al*., 2006). one possible reason of higher polymorphism of *BARE-1* or nearby DNA sequences was that *BARE-1* might have been activated recently (Vicient *et al*., 2001) and variated more,caused by different enviroments, especially to stress conditions (Kalendar *et al*., 2000).

Notably, despite our results based on the AFLP showed that loss of rare allele in the FPs (Fig. 2b), the UFPs showed relatively lower proportion of rare alleles in the SSAP analysis (Fig. 2a). It suggested that the variations of *BARE-1* or nearby sequences in different populations might be influenced by habitat fragmentation just for higher changes of *BARE-1* sequences of *H. spontaneum* in drought microhabitats (Kalendar *et al*., 2000). Taken together, the fragmented populations of *H. spontaneum* exhibited high genetic diversities and differences by two types of DNA marker of AFLP and SSAP (Table 1 and Fig. 5), and with smaller rare alleles by AFLP marker, but with more rarealleles by SSAP marker (Fig. 2). Furthermore, habitat fragmentation might has some effects on different genomic regions of wild barley, and sequences within or nearby *BARE-1* may be more sensitive to habitat fragmentation and *BARE-1* could be used as the important and useful factor to monitor environmental changes.

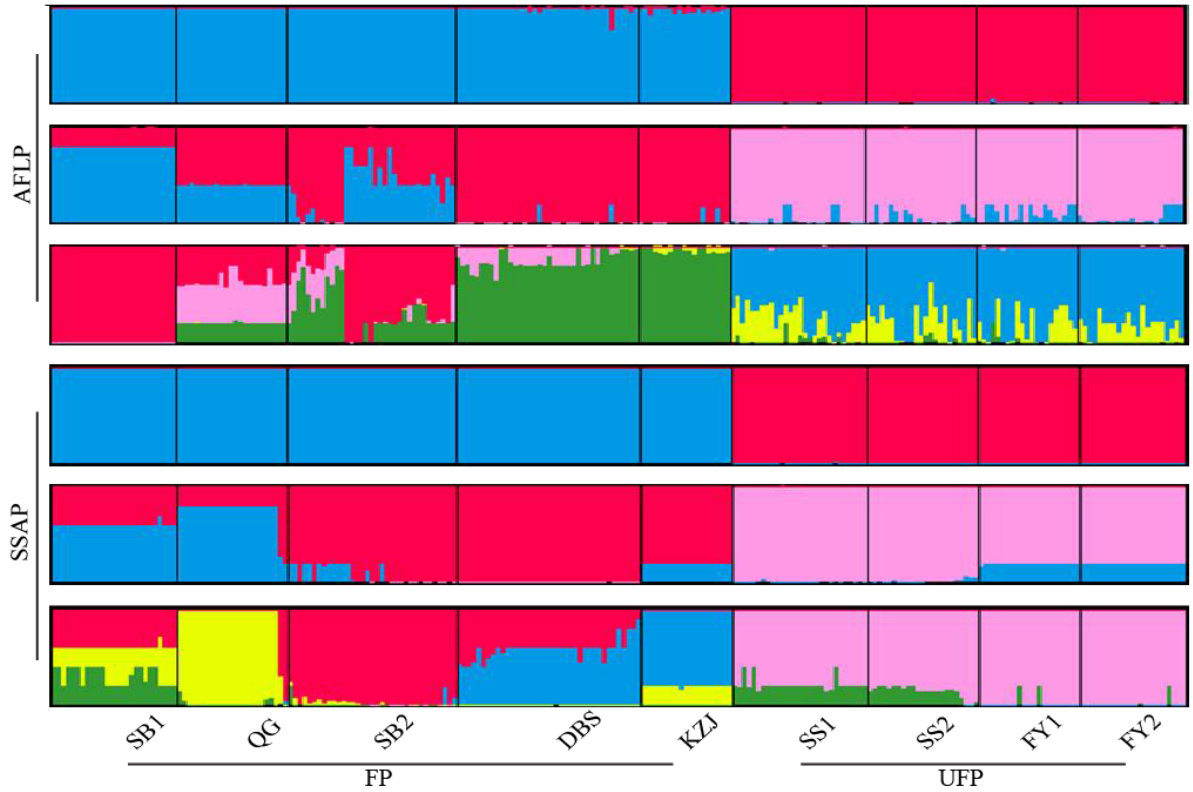

Fig. 5. District plots for STRUCTURE analysis of 9 *Hordeum brevisubulatum* populations based on AFLP and SSAP datasets. FP: Fragmented population, UFP: Unfragmented population.

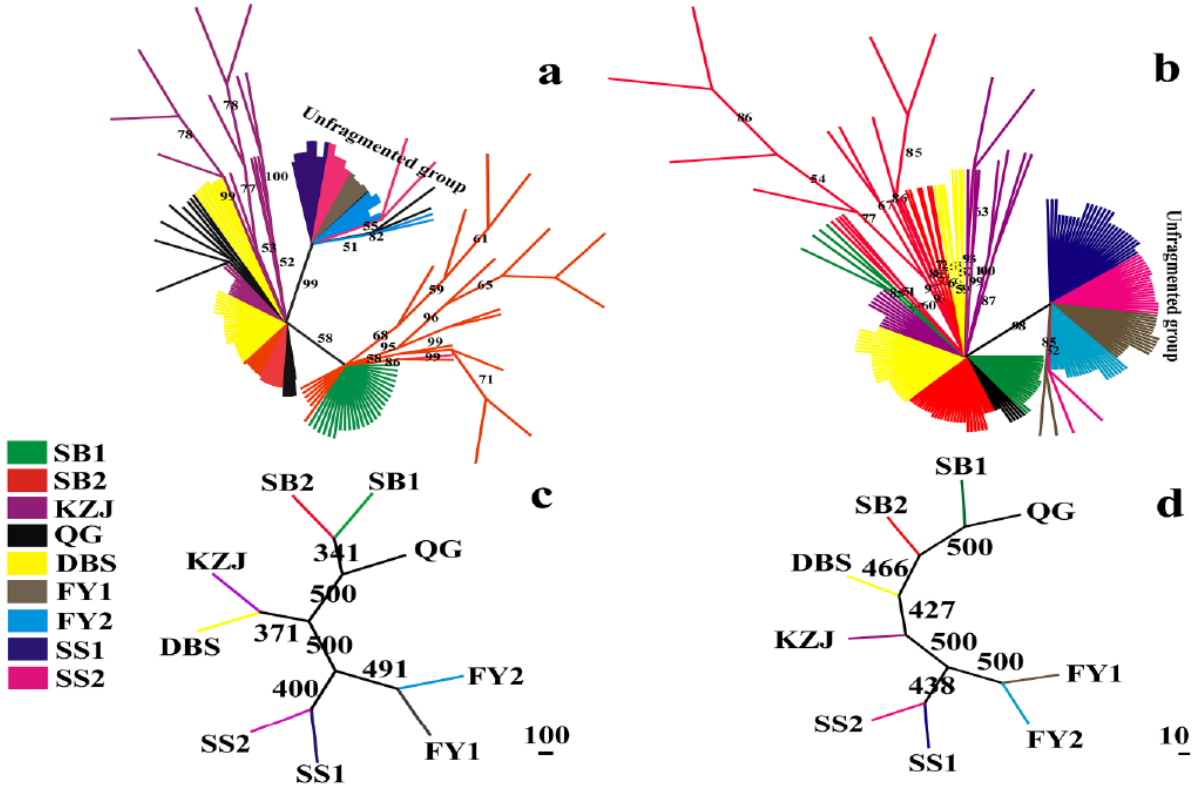

Fig. 6. NJ trees of *Hordeum brevisubulatum* with 235 individuals (AFLP (a) and SSAP (b)), and with 9 populations (AFLP (c) and SSAP (d)). Numbers indicate bootstrap support values.

Table 5. Analysis of molecular variance (AMOVA) of *Hordeum brevisubulatum*.

Marker type	sorts of populations/ Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)	*p*
AFLP	At species level					
	Among populations	8	2520.2	10.88442	24.94	<0.001
	Within populations	226	7405.3	32.76662	75.06	<0.001
	Total	234	9925.4	43.65105		
	At 2-group level (FP and UFP)					
	Among groups	1	1339	10.3546	21.4	<0.001
	Among populations within group	7	1181.2	5.2543	10.86	<0.001
	Within populations	226	7405.3	32.76662	67.73	0.0028
	Total	234	9925.4	48.37552		
SSAP	At species level					
	Among populations	8	2286.3	9.93673	26.12	<0.001
	Within populations	226	6352.5	28.10825	73.88	<0.001
	Total	234	8638.8	38.04498		
	At 2-group level (FP and UFP)					
	Among groups	1	1216.6	9.41321	22.23	<0.001
	Among populations within group	7	1069.7	4.81847	11.38	<0.001
	Within populations	226	6352.5	28.10825	66.39	<0.001
	Total	234	8638.8	42.33993		

FP: Fragmented population, UFP: Unfragmented population

Acknowledgments

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