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Comparative analyses of genetic/epigenetic diversities and structures in a wild barley species (*Hordeum brevisubulatum*) using MSAP, SSAP and AFLP

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ABSTRACT. We analyzed genetic diversity and population genetic structure of four artificial populations of wild barley (*Hordeum brevisubulatum*); 96 plants collected from the Songnen Prairie in northeastern China were analyzed using amplified fragment length polymorphism (AFLP), specific-sequence amplified polymorphism (SSAP) and methylation-sensitive amplified polymorphism (MSAP) markers. Indices of (epi-)genetic diversity, (epi-)genetic distance, gene flow, genotype frequency, cluster analysis, PCA analysis and AMOVA analysis generated from MSAP, AFLP and SSAP markers had the same trend. We found a high level of correlation in the artificial populations between MSAP, SSAP and AFLP markers by the Mantel test ($r > 0.8$).

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This is incongruent with previous findings showing that there is virtually no correlation between DNA methylation polymorphism and classical genetic variation; the high level of genetic polymorphism could be a result of epigenetic regulation. We compared our results with data from natural populations. The population diversity of the artificial populations was lower. However, different from what was found using AFLP and SSAP, based on MSAP results the methylation polymorphism of the artificial populations was not significantly reduced. This leads us to suggest that the DNA methylation pattern change in *H. brevisubulatum* populations is not only related to DNA sequence variation, but is also regulated by other controlling systems.

Key words: *Hordeum brevisubulatum*; (Epi-)genetic diversity; (Epi-)genetic structure; Gene flow

INTRODUCTION

Epigenetic variation of populations has attracted the attention of ecologists. Several studies have demonstrated that epigenetic diversity exists in plant populations (Cervera et al., 2002; Keyte et al., 2006; Lira-Medeiros et al., 2010; Yi et al., 2010). As an important epigenetic marker, cytosine methylation plays essential roles in regulating gene activity and maintaining genome integrity. Given that altered DNA methylation patterns in plants often transmit faithfully through organismal generations, and hence, potentially produce new heritable phenotypes, it is conceivable that epigenetic alleles (epialleles) in the form of altered DNA methylation patterns are important in genome evolution (Rapp and Wendel, 2005). Furthermore, because DNA methylation patterns are often responsive and prone to alterations under environmental and biological stresses (Kalisz and Purugganan, 2004; Salmon et al., 2005), it is likely that they also play an important role in coping with stress and facilitating ecological adaptation by modulating the expression of critical genes. A suitable scoring criterion for assessing polymorphisms in DNA methylation has been used in measuring epigenetic variation in populations (Keyte et al., 2006; Li et al., 2010). Henceforth, some studies have paid attention to the polymorphism of cytosine methylation patterns in populations (Herrera and Bazaga, 2010; Lira-Medeiros et al., 2010; Yi et al., 2010; Richards, 2011).

Hordeum brevisubulatum is an important grazing grass in the Songnen Prairie in northeastern China. It can live in degenerated meadows even on saline-alkali land and often constitutes locally dominant plant populations. Some researchers have cultivated this species for more than ten years. They have cultivated *H. brevisubulatum* in the settled regions without other strict rules, making it the preponderant species, where human activity is the main factor for variation of the artificial *H. brevisubulatum* populations. These *H. brevisubulatum* populations are thus appropriate and interesting materials to study the possible relationship between epigenetic variation and adaptation to environment stress.

In this study, we sampled four artificial *H. brevisubulatum* populations distributed across its ecological range in the Songnen Prairie in northeastern China, to evaluate their genetic/epigenetic variation and differentiation. Of particular interest were 1) to explore epigenetic diversity, epigenetic structure and differentiation within and between artificial popula-

tions and 2) to compare the epigenetic/genetic results to discuss contributions of epigenetic diversity for *H. brevisubulatum* populations in ecological adaptation.

MATERIAL AND METHODS

Plant material

Ninety-six individual genotypes from four artificial populations of *H. brevisubulatum* were used in this study, which distributed in a region with a latitude of 44°18' to 45°64' N and longitude of 123°11' to 124°39' E in the Songnen Prairie (Table 1). Each population was represented by 24 individuals and each individual was considered when separated from others collected by at least 1 m.

Genomic DNA was isolated from expanded leaves of individual plants by a modified CTAB method (Kidwell and Osborn, 1992) and purified by phenol extractions. In methylation-sensitive amplified polymorphism (MSAP) analysis, we chose 6 individual genotypes that were picked out respectively from the four populations on the basis of previous amplified fragment length polymorphism (AFLP) and specific-sequence amplified polymorphism (SSAP) analyses showing the most divergent patterns, and hence, they should have represented genetic diversity of each population to the fullest possible extent (Li YD, Shan XH, Guo WL and Liu B, unpublished results).

Table 1. Code, number of samples and sampling locus of *Hordeum brevisubulatum*.

Code	Samples	Sampling locus
POP1	24	45°03' N, 124°39' E
POP2	24	44°44' N, 123°44' E
POP3	24	44°18' N, 123°11' E
POP4	24	45°64' N, 124°00' E

MSAP, SSAP and AFLP analysis

For MSAP analysis, the same protocol (Cervera et al., 2002) was used. One pair of pre-selective and 8 pairs of selective primers were employed (Supplementary Table 1). For SSAP analysis, the same protocol (Waugh et al., 1997) was used. One pair of pre-selective and 11 pairs of selective primers were employed (Supplementary Table 1). For AFLP analysis, the standard protocol (Vos et al., 1995) was used with minor modifications. One pair of pre-selective and 9 pairs of selective primers were used (Supplementary Table 1). The selective amplification products of the three PCR markers were fractionated by running through 8% denaturing polyacrylamide gels for 3.5 h at 55 W, 55°C, and visualized by silver staining.

Data scoring and statistical analysis

The bands of MSAP were scored as described by Li et al. (2010). Percentage of polymorphic loci, Shannon's information index (*I*), Nei's (1973) gene diversity index (*h*), (epi-) genetic distance, relative degree of (epi-)genetic diversity (G_{ST}) and gene flow (N_m) were calculated using the POPGENE program, version 1.31 (Yeh et al., 1997). NTSYS-pc (version 2.1, Exeter software, Setauket, USA) and TFPGA1.3 (Miller, 1997) were used for Mantel tests

(Mantel, 1967) and UPGMA dendrogram construction. AMOVA (Excoffier et al., 1992) was based on Arlequin version 3.01 (Schneider et al., 2000).

RESULTS

Epigenetic variation and epigenetic structure assessed by MSAP

MSAP is usually used in detecting cytosine methylation of the genome. In this study, it was used to detect the epigenetic diversity of populations. According to the AFLP and SSAP UPGMA results (Li YD, Shan XH, Guo WL and Liu B, unpublished results), we obtained 24 individuals from each artificial populations for MSAP. These sets yielded 455 bands in artificial populations of *H. brevisubulatum*, including H/M (1/1) 25.63%, H/M (1/0) 33.78%, H/M (0/1) 16.46%, H/M (0/0) 24.13%, of which 150 (32.97%) were polymorphic. Percentage of polymorphic loci, Nei's (1973) measure of *h*, and *I* are shown in Table 2. Epigenetic structure and gene flow were analyzed, and a high variation among populations and low gene flow were found (Table 3). These results suggested that the low gene flow was the pivotal reason for the higher epigenetic variation among the artificial populations of *H. brevisubulatum*. AMOVA results indicated that most of the variation was within populations in the artificial populations of *H. brevisubulatum*. The variation within populations still accounted for the main body of total molecular variation, but this status was not significant as expected.

Table 2. Genetic diversity index within populations of *Hordeum brevisubulatum* by AFLP, SSAP and MSAP.

	Population name	Sample number	Percentage of polymorphic loci	Observed number of alleles (SD)	Effective number of alleles (SD)	Nei's gene diversity (SD)	Shannon's information index (SD)
AFLP	POP1	24	9.95	1.0995 (0.2996)	1.0724 (0.2345)	0.0400 (0.1263)	0.0581 (0.1807)
	POP2	24	6.75	1.0675 (0.2511)	1.0041 (0.0315)	0.0034 (0.0201)	0.0078 (0.0357)
	POP3	24	11.19	1.1119 (0.3155)	1.0772 (0.2371)	0.0435 (0.1289)	0.0638 (0.1857)
	POP4	24	9.41	1.0941 (0.2923)	1.0687 (0.2274)	0.0383 (0.1229)	0.0557 (0.1766)
	Mean	24	9.33	1.0933	1.0556	0.0313	0.0464
	Total	96	23.27	1.2327 (0.4229)	1.1113 (0.2627)	0.0657 (0.1474)	0.0989 (0.2151)
SSAP	POP1	24	19.65	1.1965 (0.3977)	1.116 (0.2694)	0.0681 (0.1502)	0.1019 (0.2190)
	POP2	24	12.77	1.1277 (0.3341)	1.0162 (0.0711)	0.0125 (0.0494)	0.0238 (0.0819)
	POP3	24	18.47	1.1847 (0.3884)	1.1023 (0.2568)	0.060 (0.1425)	0.0903 (0.2073)
	POP4	24	16.31	1.1631 (0.3698)	1.1012 (0.2544)	0.0594 (0.1424)	0.0885 (0.2081)
	Mean	24	16.80	1.1680	1.0839	0.0500	0.0761
	Total	96	37.72	1.3772 (0.4852)	1.1497 (0.2943)	0.0892 (0.1621)	0.1374 (0.2349)
MSAP	POP1	24	32.97	1.3319 (0.4761)	1.2075 (0.3310)	0.1225 (0.1831)	0.1832 (0.2689)
	POP2	24	22.42	1.2264 (0.4242)	1.0998 (0.2013)	0.0675 (0.1291)	0.1072 (0.2028)
	POP3	24	26.81	1.2725 (0.4555)	1.1734 (0.3174)	0.1012 (0.1747)	0.1512 (0.2570)
	POP4	24	19.56	1.1956 (0.3971)	1.1323 (0.2928)	0.0759 (0.1601)	0.1120 (0.2322)
	Mean	24	25.44	1.2566	1.1533	0.0918	0.1384
	Total	96	52.97	1.5538 (0.5522)	1.2524 (0.3462)	0.1514 (0.1845)	0.2368 (0.2700)

Table 3. (Epi-)genetic structure and change indices of *Hordeum bervisubulatum*.

Marker	N	G_{ST}	N_m
AFLP	96	0.4561	0.5230
SSAP	96	0.4394	0.6378
MSAP	24	0.3938	0.7697

G_{ST} = genetic diversity; N_m = gene flow.

Genetic variation and genetic structure assessed by SSAP and AFLP

SSAP sets yielded 509 bands in the artificial populations of *H. brevisubulatum*, of which 192 (37.72%) were polymorphic. AFLP sets yielded 563 bands, of which 131 (23.27%) were polymorphic. The results of SSAP and AFLP analyses were similar to those with MSAP (Tables 2, 3, 4, and 5). This indicated that genetic information and epigenetic information displayed similar trends in the artificial populations of *H. brevisubulatum*.

Table 4. Analysis of molecular variance (AMOVA) of all 96 individuals.

	Source of variation	d.f.	Variance components	Percentage of variation	P
AFLP	Between populations	3	8.69656	45.36	<0.001
	Within populations	92	10.47763	54.64	<0.001
	Total	95	19.17419		
SSAP	Between populations	3	11.79008	45.23	<0.001
	Within populations	92	14.27763	54.77	<0.001
	Total	95	26.06771		

d.f. = degrees of freedom.

Table 5. Comparison of AMOVA results of 24 individuals by different markers.

Source of variation	Percentage of variation		
	AFLP	MSAP	SSAP
Between populations	35.25	35.69	44.81
Within populations	64.75	64.31	55.19

Correlation between the three marker systems in populations of *H. brevisubulatum*

Based on the data of the three molecular markers, the UPGMA clustering algorithm grouped individuals into four distinct clusters; only individuals 17 and 41 were not grouped in any of the clusters, which should be due to more methylation polymorphism. Interestingly, the four clusters were completely in accordance with their populations (Figure 1 and Supplementary Figures 1 and 2). The association between the original genetic similarity matrix and the cophenetic correlation matrix was found highly significant based on the Mantel test ($r > 0.85$, 1000 permutations, $P < 0.01$), thus testifying to the authenticity of the dendrogram.

All estimates of correlation coefficients among pairwise genetic distance matrices generated by the different marker systems were calculated using the Mantel test (1000 permutations, $P < 0.01$). Any pairs of data sets of the artificial populations were significantly correlative ($r > 0.8$) (Table 6).

Table 6. Mantel test between JSI matrices of AFLP, SSAP and MSAP in populations (1000 random permutations, $P < 0.01$).

	AFLP	SSAP	MSAP
AFLP	1		
SSAP	0.88109	1	
MSAP	0.88348	0.89693	1

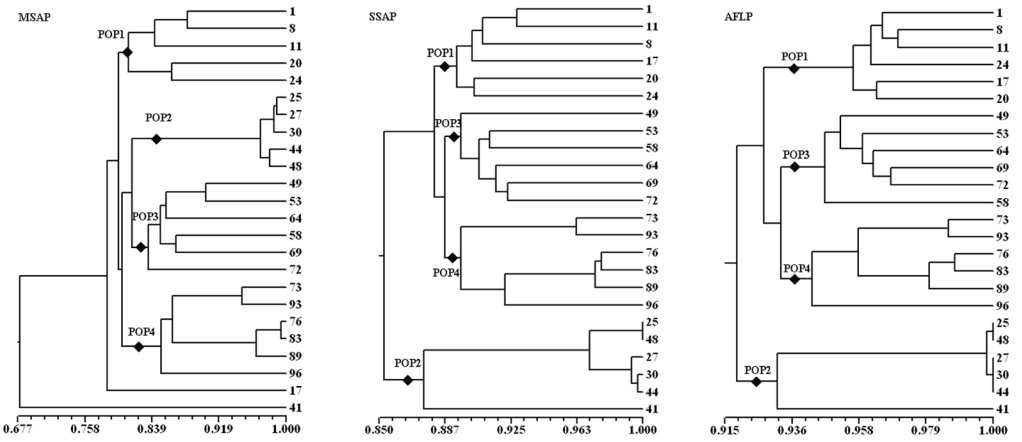

Figure 1. UPGMA dendrograms based on different marker systems, including 24 individuals.

DISCUSSION

Comparison of the effectiveness and correlation of the three PCR marker systems

SSAP and AFLP are two efficient marker systems for evaluating genetic variation and assessing genetic relationships. Especially, the retrotransposon *Bare1*-based SSAP marker has been documented as the most efficient nuclear DNA marker to detect genetic diversity in molecular ecological studies in *Hordeum* species (Waugh et al., 1997). However, MSAP has not been used in the field except in our recent studies (Li et al., 2010). In this study, we compared MSAP and the two efficient markers on the same set of *H. brevisubulatum* plants to assess the usefulness of MSAP in molecular ecological analysis of this plant species. It was found that the epigenetic diversity among the individual genotypes based on the MSAP data was higher than the genetic diversity based on the AFLP data, and even higher than with SSAP, as evidenced by the various parameters determined (Table 2). More importantly, although the relationships among the individual genotypes within a population differed to some extent between MSAP and SSAP/AFLP, the phenograms generated by the three markers were remarkably similar, and both enabled clustering of the plants into four groups largely according to their populations (Figure 1 and Supplementary Figures 1 and 2). We also found that epigenetic distance was greater than genetic distance, which implied that more epigenetic polymorphism was detected and that epigenetic variations happened more easily than genetic variations.

Epigenetic/genetic variation and epigenetic/genetic relationships in the populations of *H. brevisubulatum*

Artificial cultivation process can induce a reduction in genetic variation in plant artificial populations as compared with their natural populations. This point has been proven in many species (Papa and Gepts, 2003; Tang and Knapp, 2003; Wright et al., 2005). Our result is coincident with it. We used three molecular markers (AFLP, SSAP and MSAP) to analyze the genetic and epigenetic variation among the artificial *H. brevisubulatum*. Comparing the data of the natural *H. brevisubulatum* populations (Li YD, Shan XH, Guo WL and Liu B, unpublished results), the AFLP and SSAP results reflected a significant reduction in genetic polymorphism. By MSAP, epigenetic diversity was also detected, but it did not show the same trend as genetic diversity. One possibility is that more methylation pattern changes appeared in the artificial *H. brevisubulatum* populations, responding to abiotic stress, because these artificial populations all grow in alkali soil. A number of studies have investigated the change in epigenetic changes in the plant genome in different living conditions (Shen et al., 2006; Choi and Sano, 2007; Lukens and Zhan, 2007; Vaughn et al., 2007; Tan, 2010). In our study, we also found that percentage of DNA cytosine methylation changes in the artificial population was higher than in the natural populations (Li YD, Shan XH, Guo WL and Liu B, unpublished results). This phenomenon coincides with the conclusion of Rapp and Wendel (2005). It indicated that epigenetic diversity could be maintained at a high level to respond to some abiotic stress, while genetic diversity decreased.

Gene flow was about 3- to 5-fold higher among the natural *H. brevisubulatum* populations than that among the artificial ones. Particularly among the natural populations, the value of N_m (5.3463) was about 10-fold that of the artificial ones (N_m = 0.5230). The difference implies that cultivation impacted the gene flow among the artificial *H. brevisubulatum* populations. In wild conditions, especially on plains, there is no geographic segregation and human interference, so gene flow among populations is comparatively high, which induces low level of genetic differentiation among populations. In the artificial *H. brevisubulatum* populations, except with human selection and cultivation, the smaller area of each population and stricter isolation resulted in lower gene flow among them, which all induced genetic drift and the higher (epi-)genetic population differentiation.

In this study, we could have expected to detect a correlation between this genetic polymorphism with the DNA methylation polymorphism revealed by MSAP. Indeed, there is a high level of correlation between the markers in the artificial *H. brevisubulatum* populations (Table 6). This is incongruent with previous findings showing that there is virtually no correlation between DNA methylation polymorphism and classical genetic variations (Ashikawa, 2001; Cervera et al., 2002; Keyte et al., 2006), and further proves that epigenetic changes first respond to artificial cultivation and then affect genetic variations. This regulation may be as a result of heterogeneity in edaphic conditions, such as degrees of salinity.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. Primers used in this study.

Adapters	
*Mse*I-adapter I	5'-GACGATGAGTCCTGAG
*Mse*I-adapter II	5'-TACTCAGGACTCAT
*Eco*RI-adapter I	5'-CTCGTAGACTGCGTACC
*Eco*RI-adapter II	5'-AATTGGTACGCAGTC
H/M-adapter I	5'-GATCATGAGTCCTGCT
H/M-adapter II	5'-CGAGCAGGACTCATGA
Pre-selective primers	
*Eco*RI +A	5'-GACTGCGTACCAATTCA
H/M+0	5'-ATCATGAGTCCTGCTCGG
*Mse*I+C	5'-GATGAGTCCTGAGTAAC
*Pst*I+0	5'-GACTGCGTACATGCAG
Selective primers	
*Mse*I primers	
M-CAA	5'-GATGAGTCCTGAGTAACAA
M-CAC	5'-GATGAGTCCTGAGTAACAC
M-CAG	5'-GATGAGTCCTGAGTAACAG
M-CAT	5'-GATGAGTCCTGAGTAACAT
M-CTA	5'-GATGAGTCCTGAGTAACTA
M-CTC	5'-GATGAGTCCTGAGTAACTC
M-CTT	5'-GATGAGTCCTGAGTAACTT
M-CCA	5'-GATGAGTCCTGAGTAACCA
*Eco*RI primers	
E-ACA	5'-GACTGCGTACCAATTCACA
E-ACT	5'-GACTGCGTACCAATTCACT
E-ACC	5'-GACTGCGTACCAATTCACC
E-ACG	5'-GACTGCGTACCAATTCACG
E-AGC	5'-GACTGCGTACCAATTCAGC
E-AGG	5'-GACTGCGTACCAATTCAGG
E-AGA	5'-GACTGCGTACCAATTCAGA
E-ATC	5'-GACTGCGTACCAATTC ATC
H/M primers	
H/M-TCG	5'-ATCATGAGTCCTGCTCGGTCG
H/M-TGA	5'-ATCATGAGTCCTGCTCGGTGA
H/M-TGT	5'-ATCATGAGTCCTGCTCGGTGT
H/M-TGC	5'-ATCATGAGTCCTGCTCGGTGC
H/M-TAC	5'-ATCATGAGTCCTGCTCGGTAC
*Pst*I primers	
P1	5'-GACTGCGTACATGCAGAAT
P3	5'-GACTGCGTACATGCAGATA
P5	5'-GACTGCGTACATGCAGATG
P7	5'-GACTGCGTACATGCAGAGA
P10	5'-GACTGCGTACATGCAGACC
P11	5'-GACTGCGTACATGCAGAGG
P20	5'-GACTGCGTACATGCAGCTG
P25	5'-GACTGCGTACATGCAGCTT
P26	5'-GACTGCGTACATGCAGCGG
Bare-1	5'-CTAGGGCATAATTCCAACAA

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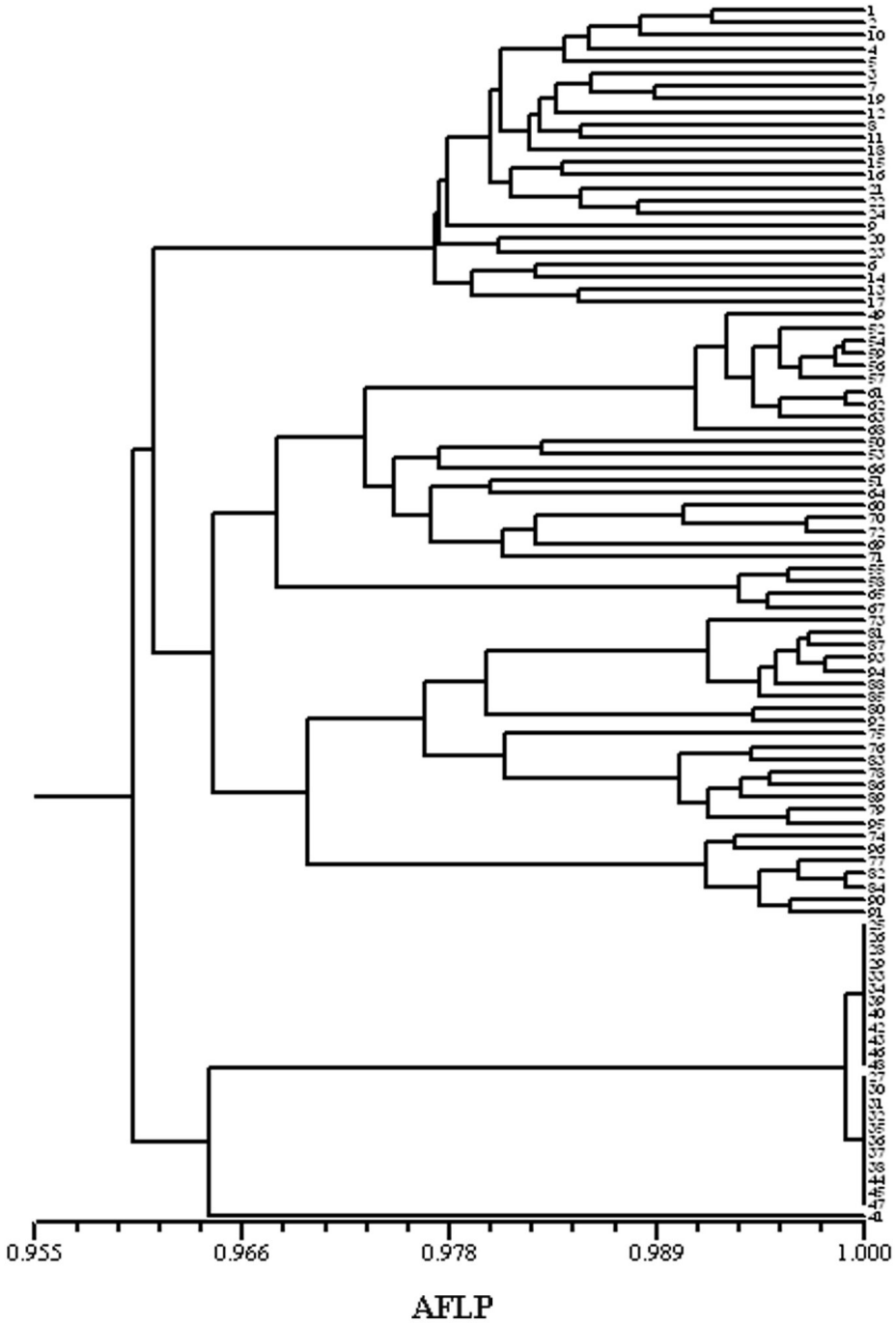
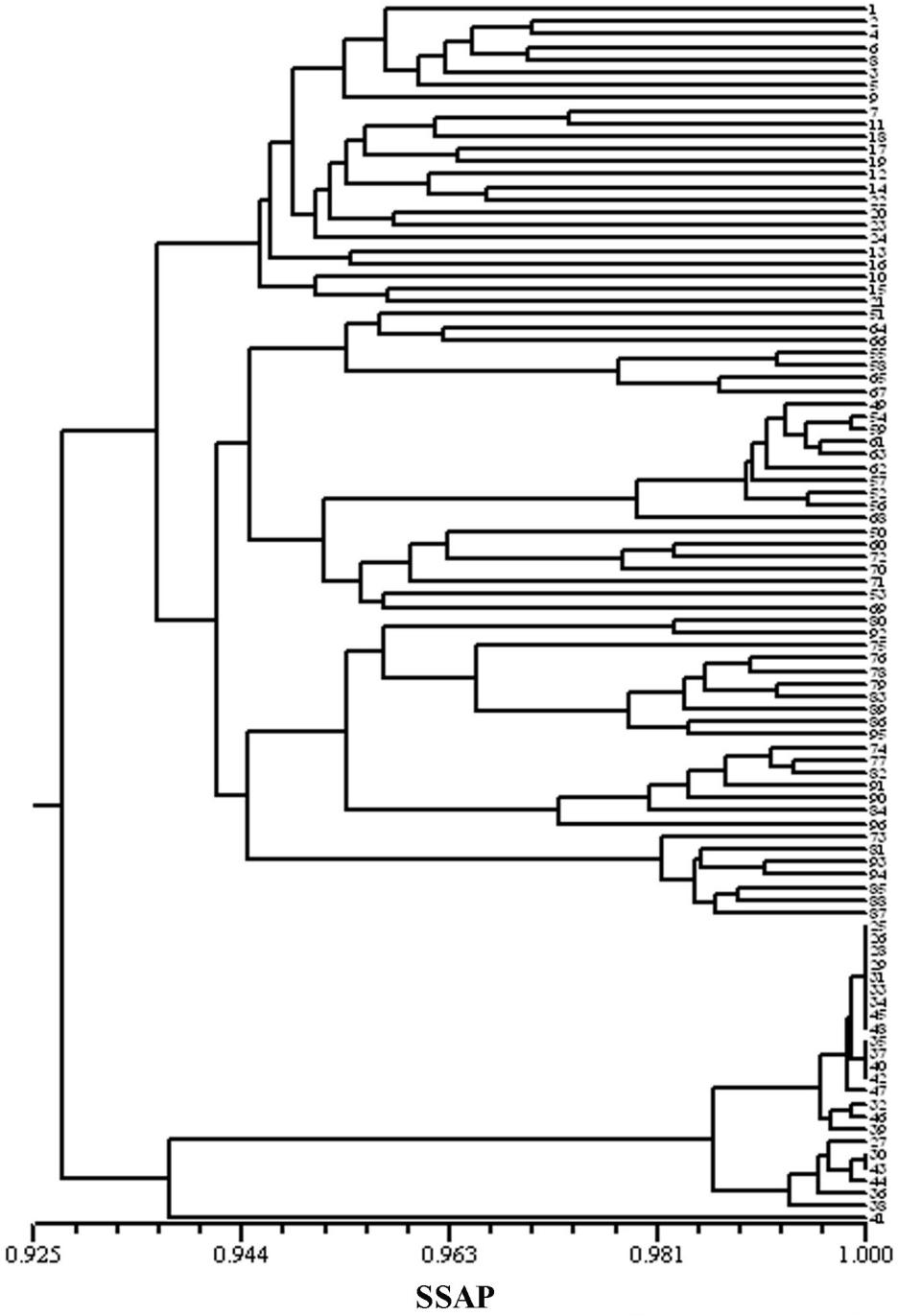


Supplementary Figure 1. UPGMA dendrograms based on AFLP, including all individuals.



Supplementary Figure 2. UPGMA dendrograms based on SSAP, including all individuals.