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Full Length Research Paper

Analysis of genetic diversity in *Arrhenatherum elatius* Germplasm using inter-simple sequence repeat (ISSR) markers

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The genetic diversity of 19 *Arrhenatherum elatius* accessions was analyzed using 100 inter-simple sequence repeat (ISSR) primers, out of which 11 generated distinct amplification products. Out of the 152 total bands detected, 107 were polymorphic. The percentage of polymorphic bands (PPB) was 68.9% with an average of 9.73 polymorphic bands per primer. The ISSR-based genetic similarity (GS) coefficients among the 19 accessions ranged from 0.4821 to 0.7411, revealing high genetic diversity. Based on the UPGMA cluster analysis and the principal components analysis (PCA), the 19 *A. elatius* accessions were divided into three groups with similar situations. We found that the genetic distance was related to the geographical distance among the 19 *A. elatius* accessions studied. These results confirm the potential value of genetic diversity preservation for future breeding programs.

Key words: *Arrhenatherum elatius*, genetic diversity, inter-simple sequence repeat (ISSR) markers.

INTRODUCTION

Arrhenatherum elatius (L.) P. Beauv. ex J. Presl is a perennial grass (Poaceae) that grows in a wide range of ecological conditions and has been introduced worldwide. *A. elatius* is of autotetraploid origin (2n=4X=28), with a few diploid exceptions (Petit et al., 1997), and is native to and widely distributed throughout Europe. It can be found in eutrophic grasslands, from low altitudes to altitudes of over 2000 m in the Alps and even higher in the Caucasus Mountains (Pfitzenmeyer, 1962). It is hypothesized that the species is not native to Central Europe but was introduced following an increase in the cultivation of grasslands at the end of the middle ages or later (Buch et al., 2007; Michalski et al., 2010) . *A. elatius* from Russia and Poland was introduced to China in the 1950's and was planted in the Sichuan, Jiangsu, Shandong and Henan provinces (Chen and Jia, 2002). *A. elatius* can grow in both the semi-arid and semi-humid areas of China (Chen and Jia, 2002; Chen et al., 1995). Previous studies have focused its botanical characteristics (Pfitzenmeyer, 1962; Chen and Jia, 2002), planting technology and productivity (Chen et al., 1995), salt resistance (Yang et al., 2006), drought resistance (Yang et al., 2011) and genetic differentiation (Michalski et al., 2010; Ducousso et al., 1990). In particular, its high phonotypic variability (Sulinowski, 1965; Mahmoud et al., 1975) and degree of genetic variation at smaller scales in populations of the species (Ducousso et al., 1990; Petit and Thompson, 1998) suggests that *A. elatius* has a strong potential for adaptive evolution. Michalski et al. (2010) investigated genetic variation at 186 AFLP (amplified fragment length polymorphism) loci in 46 European accessions of *A. elatius* and found a high genetic variability in this species.

The genetic diversity of this species has not yet been assessed by inter-simple sequence repeat (ISSR) markers. ISSR amplification is a PCR-based method that can rapidly differentiate closely related individuals and can be used as novel DNA markers for genomic fingerprinting and phylogenetic analysis in plant improvement studies (Wang et al., 2009). This technique

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Table 1. Accessions and localities of *A. elatius.*

S/N	Accession code	Locality
1	ZXY2005P-619	South of Georgia, Western Asia
2	ZXY2005P-706	South of Latvia, Eastern Europe
3	ZXY2005P-837	South of Finland, Northern Europe
4	ZXY2005P-853	Krasnodar Krai of Russia, Eastern Europe
5	ZXY2005P-877	Krasnodar Krai of Russia, Eastern Europe
6	ZXY2005P-901	North-east of Portugal, Southern Europe
7	ZXY2005P-969	North of Czechoslovakia, Central Europe
8	ZXY2005P-1021	East of Germany, Central Europe
9	ZXY2005P-1036	North of Hungary, Central Europe
10	ZXY2005P-1086	North of Hungary, Central Europe
11	ZXY2005P-1160	East of Hungary, Central Europe
12	ZXY2005P-1182	North-east of Hungary, Central Europe
13	ZXY2005P-1296	Crimea of Ukraine, Eastern Europe
14	ZXY2005P-1319	Central of Poland, Central Europe
15	ZXY2005P-1362	Central of Poland, Central Europe
16	ZXY2005P-1375	Central of Poland, Central Europe
17	ZXY2005P-1426	West of Kirghizia, Central Asia
18	ZXY2005P-1473	West-central of White Russia, Eastern Europe
19	ZXY2005P-1514	Kiev-Region of Ukraine, Eastern Europe

utilizes a single primer composed of a microsatellite sequence anchored at the 3′ or 5′ end by 2 to 4 arbitrary and often degenerated nucleotides, and it does not require prior knowledge of the DNA sequence for primer design (Wang et al., 2009). It has the potential to be widely used, especially for evaluating plant germplasm and genetic diversity (Liu and Cordes, 2004). The objectives of this investigation were to study the genetic diversity and the effect of geographical distance on the genetic diversity of *A. elatius* using ISSR molecular markers, to provide evidence for genetic improvement, and to evaluate the applicability of this analysis for assessing phylogenetic relationships.

MATERIALS AND METHODS

Study accessions

A total of 19 accessions of *A. elatius* were used in this study. They were introduced from 12 countries, including Russia, Hungary, Germany, Finland and Poland, etc, by Beijing Institute of Animal Science, Chinese Academy of Agricultural Science (CAAS), in 2005, and the seeds were provided by CAAS in 2009 (Table 1).

DNA extraction and ISSR-PCR

DNA extraction was performed on young leaves of each accession by the cetyltrimethyl ammonium bromide (CTAB) method with a minor modification. DNA quality and quantity was checked by 1.0% agarose gel electrophoresis and spectrophotometry, respectively.

The PCR reaction was carried out in 25 µl of a mixture containing 50 ng of genomic DNA, 2.5 mM of $MgCl_2$, 0.3 mM of dNTP, 0.4 µM of primer, 1.5 U Taq DNA polymerase, and 1× buffer. The PCR cycling conditions were as follows: 94ºC for 7 min (initial denaturation), followed by 43 cycles of 94ºC for 30 s (denaturation), 53ºC for 45 s (annealing), and 72ºC for 2 min (extension), with a final 7 min extension at 72ºC and cool down to 10ºC.

DL 2000 ^{TM} DNA Marker was purchased from Dalian Takara Biotechnology Co., Ltd., of China. 100 ISSR primers from Beijing Solarbio Science & Technology Co., Ltd., of China were screened in this study. The 11 ISSR primers that gave clear and polymorphic bands were used in subsequent experiments (Table 2). Amplifications were repeated twice for each primer analyzed. PCR products were separated on 6% non-denaturalization polyacrylamide gels for 3 to 4 h at 260 V. DNA fragments were visualized by silver staining. The silver staining procedure consisted of 6 min in a solution of 9.5% ethanol and 0.5% acetic acid, staining for 12 min in a 0.2% (w/v) silver nitrate solution, rinsing for 3 min in deionized water, and development in a solution of 1.5% (w/v) sodium hydroxide (NaOH), 0.58% (w/v) formaldehyde, and 0.002% (w/v) sodium thiosulfate until the bands became visible (Zhang et al., 2002). They were photographed under white light using a PD-HA X-ray film viewer (Guangdong Yuehua Medical Instrument Factory Co., Ltd., of China).

Data analysis

The ISSR bands were scored using a binary scoring system that recorded the presence or absence of bands as 1 or 0, respectively. Genetic similarity (GS) values were calculated according to Nei's method, (Nei, 1978), that is:

$$GS = \frac{2N_{ij}}{N_i + N_j}$$

Table 2. Sequences and results of the 11 effective primers.

Primer number	Sequences (5'-3')	Total number of band	Number of polymorphic band	The percentage of polymorphic band (%)
810	GAG AGA GAG AGA GAG AT	16	13	81.3
827	ACA CAC ACA CAC ACA CG	13	8	61.5
835	AGA GAG AGA GAG AGA GYC	16	11	68.8
840	GAG AGA GAG AGA GAG AYT	23	18	78.3
843	CTC TCT CTC TCT CTC TRA	13	10	76.9
876	GAT AGA TAG ACA GAC A	6	3	50.0
879	CTT CAC TTC ACT TCA	16	11	68.8
881	GGG TGG GGT GGG GTG	14	9	64.3
886	VDV CTC TCT CTC TCT CT	13	8	61.5
899	CAT GGT GTT GGT CAT TGT TCC A	12	8	66.7
900	ACT TCC CCA CAG GTT AAC ACA	10	8	80.0
Sum		152	107	
Mean		13.82	9.73	68.9

Where, N_{ij} is the amplified mutual band numbers between accession i and accession j, N_i is the amplified band number of accession i and N_j is the amplified band number of accession j.

Data analyses were performed using the computer program NTSYSpc version 2.1. Similarity values were used to generate a dendrogram via the unweighted pair group method with arithmeticaverage (UPGMA). A principle components analysis (PCA) was also performed.

RESULTS

Polymorphisms of ISSR markers

Eleven (11) of the 100 ISSR primers used yielded banding patterns that were clear and could be scored with confidence (Table 2). Five of the 11 primers (810, 827, 835, 840 and 876) consisted of “A+G” and “A+C” repeat motifs. Satisfactory results in terms of band resolution were obtained by using primers with different 2-bases. The genome of *A. elatius* had many simple sequence repeats, such as “AC”, “CA” and “GA”.

In order to get the greatest resolution, the PCR products were also separated on 6% non-denaturalization polyacrylamide gels. Figure 1 shows the bands amplified by primer 810 and 879 on 6% non-denaturalization polyacrylamide gels. Clearly detectable and reproducible bands ranged from 150 to 1800 bp in size. The 11 ISSR primers produced a total of 152 bands, of which 107 (68.9%) were polymorphic, with an average of 9.73 polymorphic bands per primer (Table 2).

Genetic diversity of *A. elatius*

Genetic diversity analysis revealed that the genetic similarity (GS) coefficients among the 19 *A. elatius* accessions ranged from 0.4821 to 0.7411. The highest similarity of 0.7411 was observed between No. 4 and 5, both of which were from Krasnodar Krai of Russia, whereas the lowest similarity of 0.4821 was observed between No. 2 (south of Latvia) and 13 (Crimea of Ukraine), No. 4 and 9 (north of Hungary), No. 5 and 7 (north of Czechoslovakia), and No.7 and 11 (east of Hungary) (Table 3).

A dendrogram was generated using UPGMA based on the GS coefficients among the 19 accessions of *A. elatius* to show their genetic relationships (Figure 2). Three groups were clustered at a GS coefficient of 0.58. Group I consisted of 13 accessions divided into 3 sub-groups at a GS coefficient of 0.62. The first sub-group consisted of 10 accessions divided into 3 small groups at a GS coefficient of 0.64, the small group A included the 4 accessions of No. 1, 13, 17 and 19; the small group B is formed by the 5 accessions No. 4, 5, 14, 15 and 16; and the small group C consisted No. 8 accession. The second sub-group contained accessions No. 6 and 11. The third sub-group was only No. 12 accession. Group II consisted of 3 accessions divided into 2 sub-groups at a GS coefficient of 0.62, the first sub-group consisted of only No. 2 accession and the second sub-group consisted of No .7 and 18 accessions. Group III consisted of 3 accessions divided into 2 sub-groups at a GS coefficient of 0.67, the first sub-group consisted only No. 3 accession and the second sub-group consisted of No.9 and 10 accessions.

The first two axes of the principal component analysis (PCA) of the 19 accessions of *A. elatius* are shown in Figure 3. PCA divided the accessions into three groups which was similar to the dendrogram derived by UPGMA. The genetic distance correlated with the geographical distance among the accessions studied; the greater the genetic distance, the larger the geographical distance.

DISCUSSION

Genetic diversity within a species is useful for illustrating the adaptive evolution and the new variety breeding.

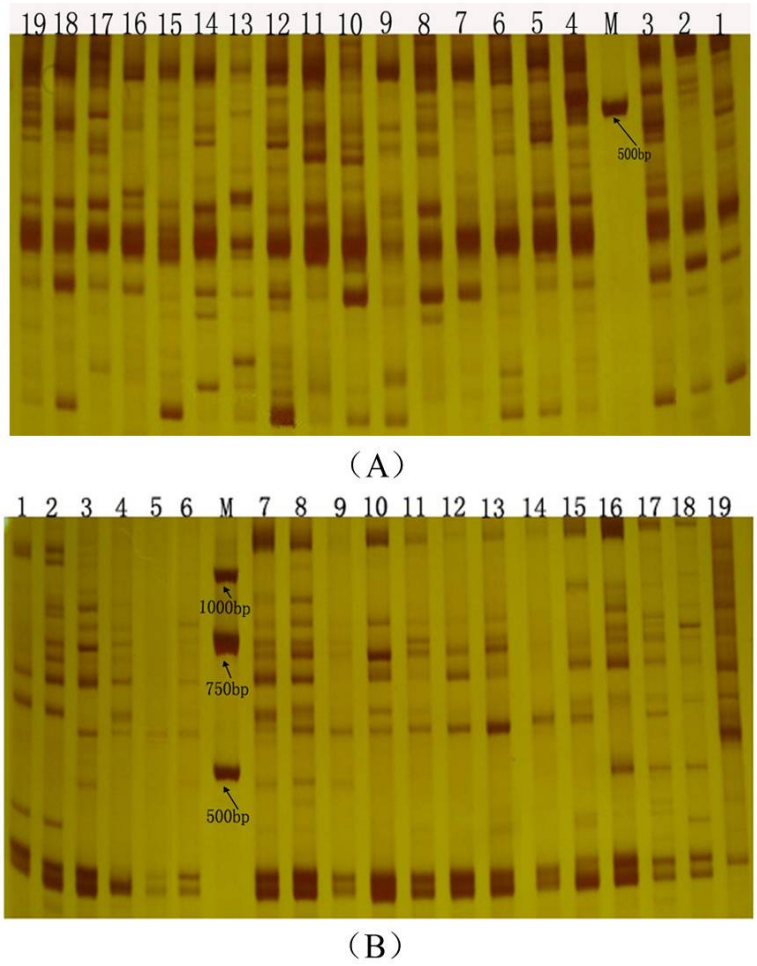

Figure 1. ISSR band profile of primer 810 (A) and primer 879 (B) on 6% non-denaturalization polyacrylamide gels. M, DNA marker. Lanes 1 to 19: See Table 1 for *A. elatius* accessions codes.

Traditional identification and analysis of genetic diversity was based on morphological characteristics and required long and careful observation of the plants in the field, which could easily be effected by developmental stage, environmental and cultivation conditions, and sampling errors. Biochemical markers such as isozymes, have been used to study the plant genome, but they have some disadvantages. Currently, there are many molecular marker methods, such as random amplification of polymorphic DNA (RAPD), simple sequence repeat (SSR), AFLP, sequence-related amplified polymorphism (SRAP) and ISSR, that are more practical ways to analyze plant genetic diversity (Zhou, 2005).

ISSR-PCR analysis is useful for studies concerning genetic diversity, either at the intra- or inter-specific level, phylogenies and evolutionary biology. ISSR markers are highly reproducible due to their primer length, very stringent due to the annealing temperature, and provide detailed polymorphic fingerprints (Kojima et al., 1998; Bornet and Branchard, 2001). ISSR markers have been successfully used for the assessment of genetic diversity in *A. elatius* (Michalski et al., 2010), *Iris lactea* (Wang et al., 2010), common bean (Galvan et al., 2003), *Vicia faba* (Terzopoulos and Bebeli, 2008) and others. In this study, we utilized ISSR-PCR analysis to detect genetic diversity in the 19 *A. elatius* accessions. Eleven (11) of the ISSR primers produced 152 discernible DNA fragments, 107 of which were polymorphic. Our results revealed that there were relatively high numbers of polymorphisms in the 19 accessions of *A. elatius* based on statistical data, and the average percentage of polymorphisms was about 68.9% with approximately 9.73 polymorphic bands per primer. High genetic diversity was previously shown in 46 European *A. elatius* accessions by AFLP markers, with 437 individuals from 46 accessions scored at 186 AFLP loci, and the proportion of polymorphic loci and the genetic diversity (determined by Jaccard dissimilarity) ranged from 64.5 to 84.9% and from 0.264 to 0.437, respectively (Michalski et al., 2010). The genetic diversity of *A. elatius* (determined by Jaccard dissimilarity) was

Table 3. Genetic similarity coefficients for the 19 accessions of *A. elatus* based on ISSR analyses

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.0000																		
2	0.6071	1.0000																	
3	0.5982	0.5268	1.0000																
4	0.6518	0.6518	0.5357	1.0000															
5	0.6607	0.6250	0.5804	0.7411	1.0000														
6	0.5982	0.5268	0.5357	0.6071	0.6161	1.0000													
7	0.5536	0.5893	0.4911	0.5089	0.4821	0.5804	1.0000												
8	0.5982	0.5625	0.5536	0.6429	0.5982	0.5536	0.5625	1.0000											
9	0.5893	0.5536	0.5982	0.4821	0.5446	0.5268	0.5893	0.5536	1.0000										
10	0.5893	0.5179	0.5625	0.5268	0.5357	0.5804	0.5357	0.5089	0.6696	1.0000									
11	0.6250	0.5893	0.5268	0.5982	0.6250	0.6161	0.4821	0.5446	0.5714	0.6250	1.0000								
12	0.5893	0.5714	0.5268	0.5446	0.5714	0.5804	0.5714	0.4911	0.5446	0.5893	0.6071	1.0000							
13	0.7143	0.4821	0.5625	0.6339	0.6250	0.5446	0.5357	0.5982	0.6161	0.6250	0.5893	0.6250	1.0000						
14	0.5982	0.5625	0.5893	0.6250	0.7054	0.6071	0.5804	0.5893	0.5179	0.5982	0.5446	0.6339	0.6518	1.0000					
15	0.5804	0.5982	0.5536	0.6607	0.6696	0.6429	0.5625	0.6429	0.5714	0.5268	0.6339	0.5625	0.6161	0.6964	1.0000				
16	0.5804	0.5625	0.5714	0.6071	0.6339	0.5714	0.5804	0.6429	0.5000	0.5625	0.5804	0.6161	0.6339	0.6964	0.7321	1.0000			
17	0.6696	0.5625	0.5536	0.6429	0.6161	0.6250	0.5268	0.6071	0.5893	0.5446	0.6339	0.6161	0.6696	0.6071	0.6607	0.7143	1.0000		
18	0.5714	0.6071	0.5804	0.5268	0.5536	0.5982	0.6250	0.5982	0.6429	0.5893	0.5714	0.5893	0.6250	0.5982	0.5982	0.6339	0.6696	1.0000	
19	0.6786	0.5536	0.5804	0.5982	0.6250	0.5982	0.5000	0.6161	0.5804	0.5714	0.5714	0.6786	0.6429	0.6518	0.6161	0.6518	0.6875	0.5714	1.0000

found to be similar to or even higher than other xenogamous grasses, including *Lolium perenne*, *Uniola paniculata* and *Poa arachnifera* (Michalski et al., 2010; Rolden et al., 2000; Renganayaki et al., 2001).

The dendrogram obtained by the UPGMA method according to the GS coefficients for the 19 *A. elatius* accessions consisted of three major groups (Figure 2). Group I included 13 accessions, all of which originally came from Central and Eastern Europe, except for No. 6 (Portugal, Southern Europe), No. 1 (Western Asia) and No. 17 (Central Asia). In particular, in Group I, No. 4 and 5 accessions from Krasnodar Krai of Russia were clustered, No. 14, 15 and 16 accessions from central Poland were clustered, and No. 1 and 17 accessions from West and Central Asia, and No. 13 and 19 from Ukraine in Eastern Europe were clustered together first, respectively. Also, No. 6 accession from northeast of Portugal in Southern Europe and No. 11 from north of Hungary in Central Europe were clustered together first and then subsequently clustered with No. 12 accession from northeast of Hungary in Central Europe. Group II consisted of No. 2, 18 and 7 accessions from Latvia and White Russia in Eastern Europe and Czechoslovakia in Central Europe, respectively. Group III was formed by 3 accessions, including No. 3 accession from south of Finland in Northern Europe and No. 9 and 10 accessions from north of Hungary in Central Europe. The results from the PCA based on the ISSR polymorphisms in the 19 accessions were similar to those from the dendrogram derived by UPGMA and support the clusters obtained by UPGMA. PCA also revealed that the genetic distance correlated with geographical distance in the 19 accessions studied.

A previous study (Michalski et al., 2010) showed that 46 European accessions of *A. elatius* could be separated into a Western European group and a Central/Eastern European group, and that only

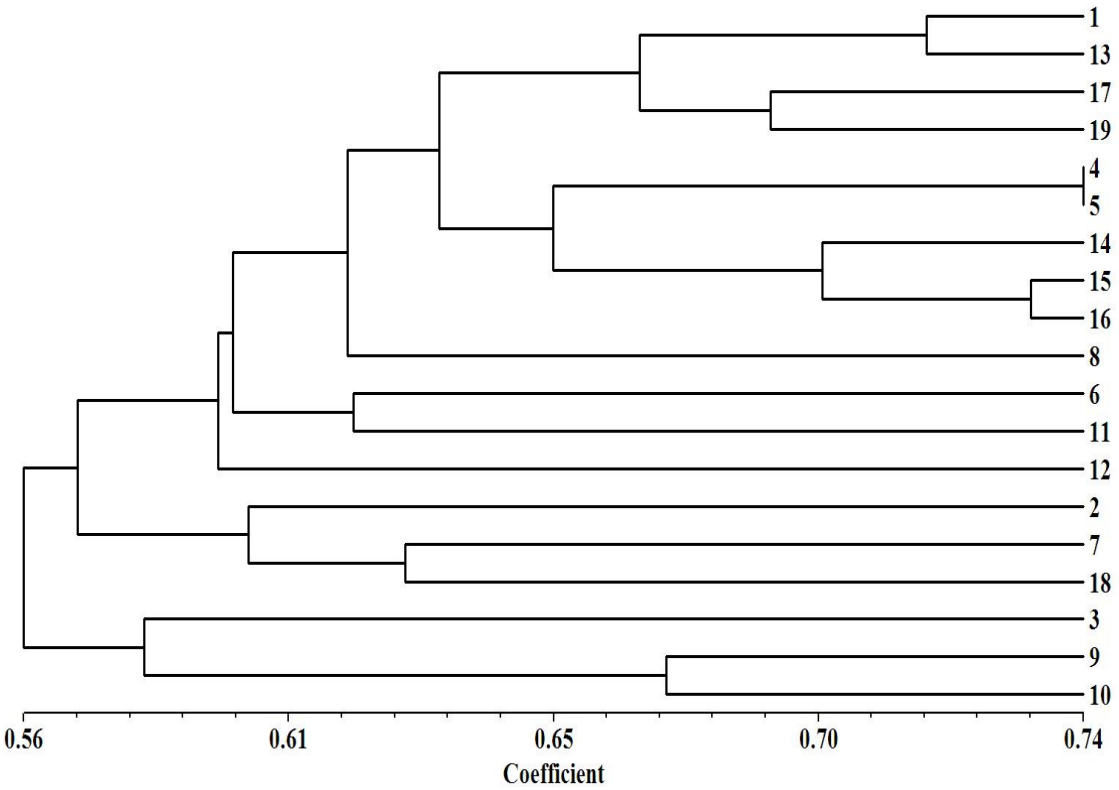

Figure 2. UPGMA-derived dendrogram of the 19 accessions based on genetic similarity coefficients (See Table 1 for *A. elatius* accessions codes.).

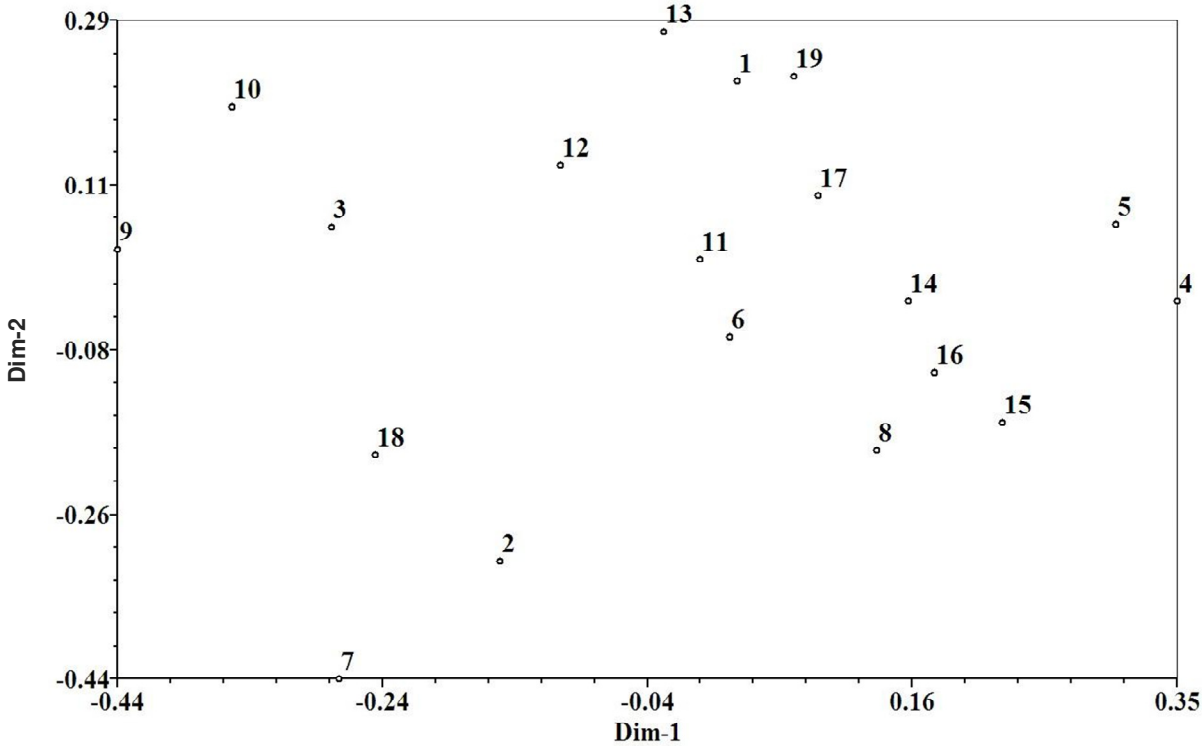

Figure 3. Principle component analysis (PCA) based on ISSR polymorphisms in the 19 accessions (See Table 1 for *A. elatius* accessions codes.).

the Western European accessions formed a larger, well supported dendrogram, while the Central/Eastern European accessions remained without a clear structure, possibly due to human activity. We also used the ISSR method to analyze the genetic diversity of *A. elatius* accessions, but the 19 accessions in our study were not only collected from Central and Eastern Europe (15 accessions), but also from Asia, Northern Europe and Southern Europe (4 accessions). The distribution of genetic diversity within and among populations is largely governed by life-history traits and historical processes affecting gene exchange between individuals (Pannell and Dorken, 2006). The genetic diversity of *A. elatius* accessions also correlates with the number of the growing days (Michalski et al., 2010). The correlation between genetic and geographical distances among *A. elatius* accessions needs to be further studied using more accessions across greater geographic distances to determine whether it is due to human activity or other reasons.

In conclusion, this study demonstrated the high genetic diversity in *A. elatius* germplasm by using ISSR markers. We found that genetic distance correlated to geographical distance in the 19 *A. elatius* accessions studied.

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