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GENETIC DIVERSITY OF WILD *MEDICAGO SATIVA* BY SEQUENCE-RELATED AMPLIFIED POLYMORPHISM MARKERS IN XINGJIANG REGION, CHINA

MA JIN-XING[1,2] , WANG TIE-MEI[1], AND LU XIN-SHI[1*]

[1]*College of Forestry, Beijing Forestry University, Beijing 100083 P.R. China*
[2]*National Animal Husbandry Service, Ministry of Agriculture of the People's Republic of China, Beijing 100125 P.R. China*
[*]*Corresponding author: Prof. Lu Xin-Shi, e-mail: luxinshi304@126.com*

Abstract

SRAP (Sequence-related amplified polymorphism) molecular markers were used to detect the genetic diversity of 22 accessions of wild *Medicago sativa* from Xinjiang. Studying on the 22 accessions 15 pairs of primer combinations detected a total of 252 bands, and each pair of primers amplified 12 polymorphic bands in average. The percentage of polymorphic bands (PPB) was 78.68%.The Nei's genetic similarity coefficient of the tested accessions ranged from 0.182 to 0.857 and the average Nei-Li's coefficient was 0.621. These results suggested that there were rich genetic diversity with the Beijiang resources comparing to Nanjiang resourses. The result from the UPGMA cluster analysis indicated that genetic diversity of wild *Medicago* could be divided into 5 groups in Xinjiang region and it is of great beneficial to protect alfalfa germplasm resources for developing local husbandry.

Introduction

With the increasing pressure of global climate change and land use in pastoral areas, the expansion of endemic species is considered to be encountered in the both environment and human threatens (Dong *et al*., 2011). To maintain genetic diversity within populations is seen as the primary goal of the conservation work now, because it will increase the threatened species to adapt to future environmental change (Julier *et al.*, 2003; Jump & Peñuelas, 2005). A preservation of natural populations and utilization its germplasm resources successfully require a good understanding of their genetic variation pattern (Pressoir & Berthaud, 2004; Badri *et al.*, 2008).

Medicago sativa (2 n = 4 x = 32) is one of the most important forage legume with over 32 million hectares in the world and is believed to have originated in the Caucasus region: northeastern Turkey, Turkmenistan and northwestern Iran (Michaud *et al*., 1988). Crop improvement depends largely on the availability of diverse germplasms and their efficient utilization. Thus, the analysis of genetic variability within and among populations of cultivated alfalfa can assess future risk of genetic erosion and help in the development of sustainable conservation and genetic improvement strategies (Stanford, 1951; Flajoulot *et al*., 2005).

In the present study, studies on the genetic diversity of *Medicago sativa* mainly focused on its morphological and yield traits, histological traits, molecular marker and allozyme markers (Li *et al*., 2009). But the molecular marker plays pivotal role in this regard, which are effective methods for detecting genetic diversity and population structure (Englbrecht *et al.*, 2000; Whitehead *et al.*, 2003). Thus, many different molecular marker have been used to analysis genetic diversity in *Medicago sativa* . Such as RFLP (Kidwell *et al.*, 1999; Maureira *et al.*, 2004), RAPD (Crochemore *et al.*, 1996; Gherardi *et al.*, 1998; Musial *et al.*, 2002; Tucak *et al.*, 2008; Marzouk & El-Bakatoushi, 2011; Rasool, 2013), SSR (Flajoulot *et al.*, 2005; Falahati-anbaran *et al.*, 2007; Touil *et al.*, 2008), SRAP (Vandemark *et al.*, 2005) and AFLP (Segovia-Lerma *et al.*, 2003). In this study, we analyzed the intra and inter-population genetic variation of *Medicago sativa* using SRAP markers, which were used in the study of plant genetic diversity in recent years (Meng *et al.*, 2012). This study aims to (1) investigate the level of genetic diversity of wild *M. sativa* populations; (2) further providing useful information for the development of appropriate germplasm management strategies.

Materials and Methods

Plant collection: *Medicago sativa* seeds were randomly sampled from different geographical region in Xinjiang Uigur Autonomous Area, China. The information about the collections of *Medicago sativa* was shown in Fig. 1. All plants for this study were raise from seed and grown under glasshouse of Beijing Forestry University (Beijing, China). Fresh leaf tissue of twenty-two individuals of each accession was bulked for DNA extraction.

DNA extraction: Genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) procedure of Doyle & Doyle (1987). DNA concentration and purity were determined with UV–vis spectrophotometer and the quantity of DNA was determined by 1% (w/v) agarose gels. The solution was diluted to 20 ng/ml and both the diluted solution and mother liquor were stored at -20^{o}C for use.

SRAP-PCR amplification: Fifteen primers were synthesized by Beijing Aoke Biological Engineering Technology Co., Ltd. Primer sequence was shown in Table 2. PCR amplification reaction system with total volume of 25μl contained 1μl DNA (20ng/μl), 0.6μl Taq DNA polymerase (2.5U), 1.4μl dNTPs 0.2mmol/L), 2μl primer(0.4μmol/L), 2.5μl 10 × Buffer (contain Mg^{2+}) and 18.9μl ddH_2O. PCR amplification was carried out on Biometra T-Gradient Thermoblock instrument and subjected to the following profile: 5 min of denaturing at 94^{o}C, 5 cycles of three steps: 1 min of denaturing at 94^{o}C 1 min of annealing at 35^{o}C, and 1 min of extension at 72^{o}C. In the following 35 cycles, 1 min of denaturing at 94^{o}C, 1 min of annealing at 50^{o}C, 1 min of extension at 72^{o}C, ending with a final extension of 7 min at 72^{o}C and conservation at 4^{o}C. PCR products were separated on 6% denatured polyacrylamide gel and detected by silver dyeing. Then, clearly and reproducibly distinguished bands were recorded and used in the following analysis.

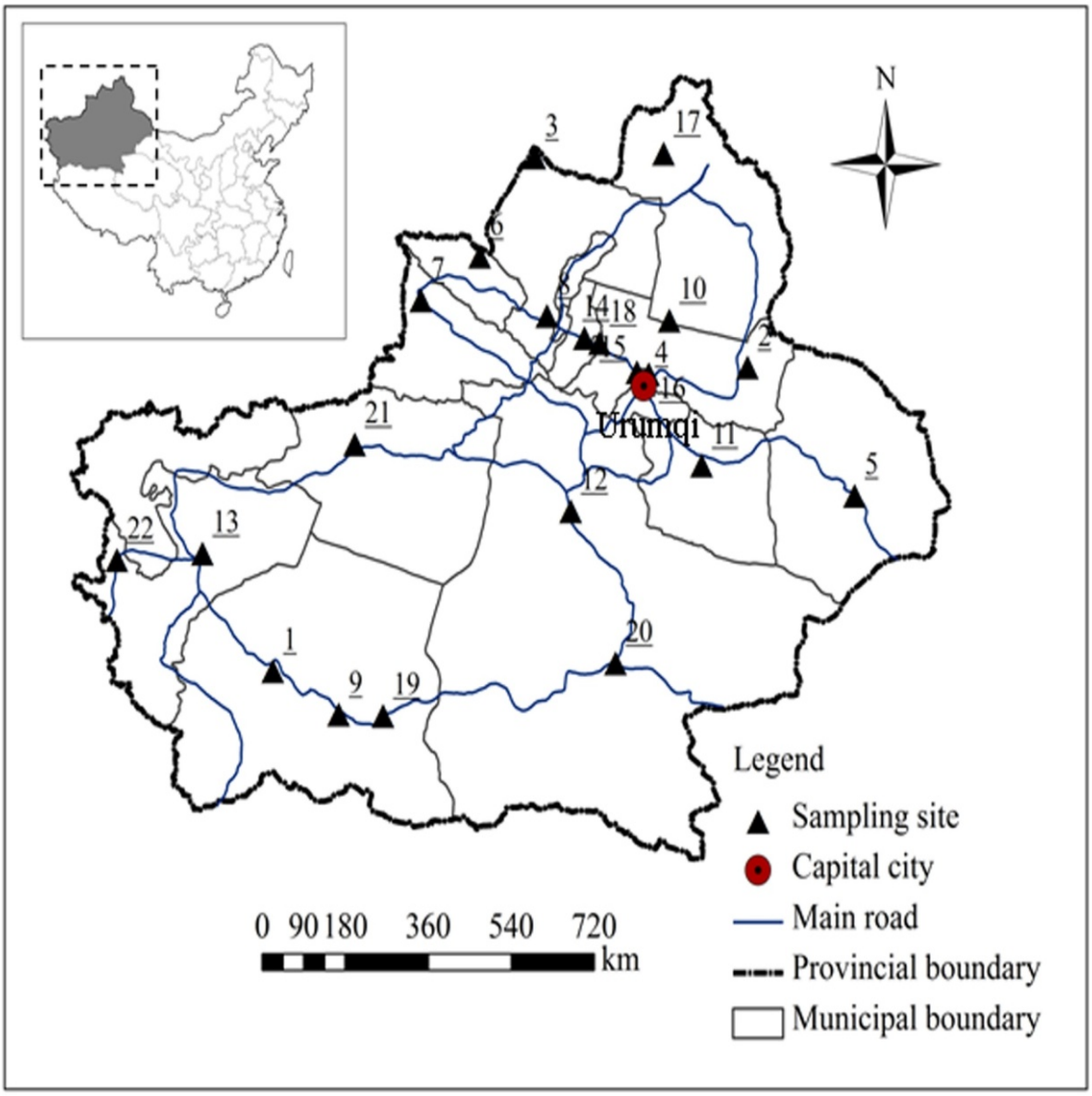

Fig. 1. Names and geographic distribution of *Medicago sativa* sources used in this study (1, 2044; 2, 2713; 3, XJ-079; 4, 2712; 5, 2723; 6, 2724; 7, 2726; 8, 2776; 9, 2777; 10, 2778; 11, 2779; 12, 2780; 13, 2781; 14, 2782; 15, 2783; 16, 2784; 17, 2785; 18, 2786; 19, 2787; 20, 2788; 21, 2789; 22, 2790).

Data analysis: Clear bands were selected for statistical analysis which ranging in size from 100 ~ 2000 bp. Amplified fragments was scored for each individual as presence (1) or absence (0). POPGENE1.32 (Yeh & Boyle, 1997) was used to calculate (G_{st}). An UPGMA (Unweighted Pair-group Method using Arithmetic mean) dendrogram was constructed NTSYS-pc (Rohlf, 2000). The structure of genetic variation among the accession was analyzed using a principal component analysis (PCA) were calculated by SPSS11.0 (Anon., 2001).

Results

An informative and reproducible amplification of SRAP fragments was established using the 15 primers (Fig. 2, Table 1).

Genetic variability details from SRAP markers: Of the 22 alfalfa germplasm accessions tested with 15 random selected primers, 252 polymorphic bands were produced (Table 2). Among these bands, 180 polymorphic loci (16.8 loci per arbitrary primer) were identified ranging from 100 to 2000 bp. The average number of polymorphic markers and the percentage of polymorphism were 12 and 78.68% respectively. The numbers of bands and polymorphic bands were produced by each primer varied. The highest number of bands (44) were produced by the primer F12-r9 and the lowest number of bands (2) by primer F16-r15. F12-r9 and F16-r14 were the most polymorphic baring 28 and 25 bands, respectively. Whereas F9-em5 and F16-r15 were the least polymorphic.

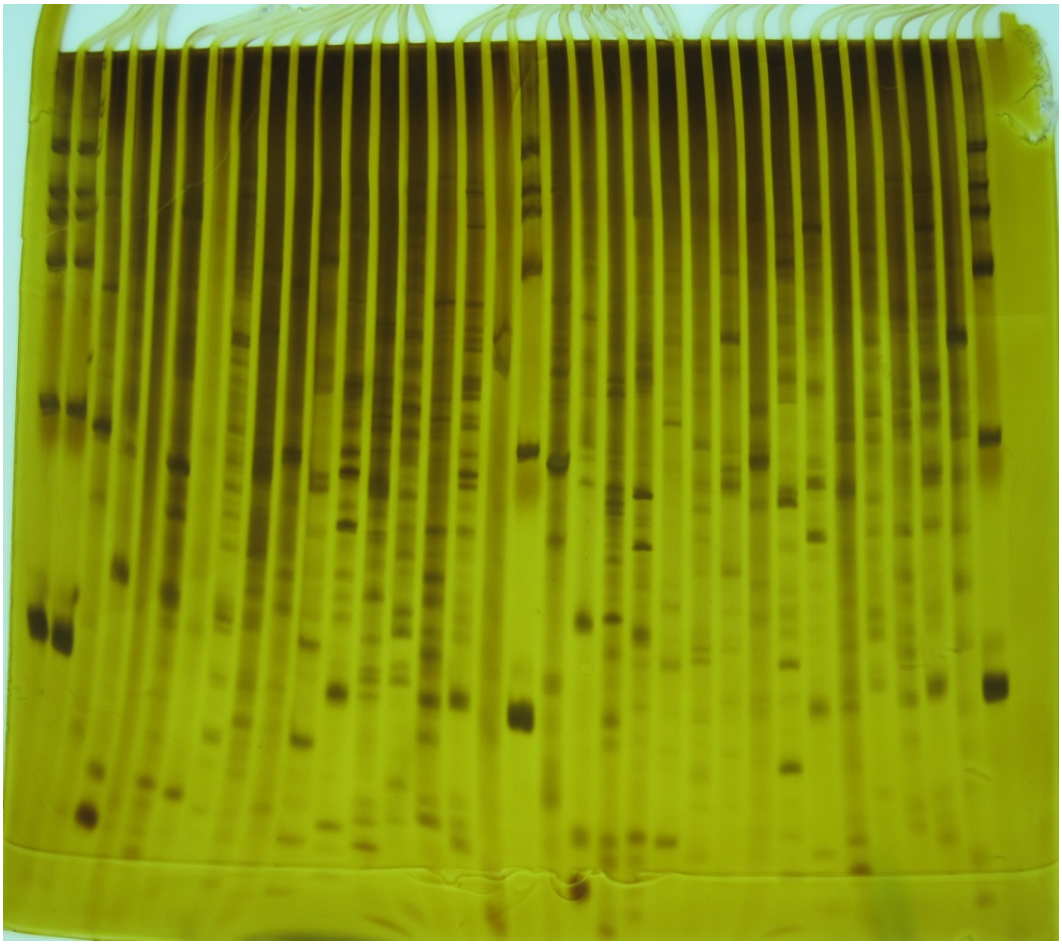

Fig. 2. Results of polyacrylamide gelelectrophoresis for *Medicago sativa* in this study.

Table 1. Description of primers sequences used for SRAP analysis of *Medicago varia* Martin.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
F12-r15	F: 5′-CGAATCTTAGCCGGAGC-3′ R: 5'- CGCACGTCCGTAATTCCA-3′	Me4-em4	F: 5′-TGAGTCCAAACCGGACC-3′ R: 5′-GACTGCGTACGAATTTGA-3′
F14-r14	F: 5'-CGAATCTTAGCCGGAAT-3′ R: 5'- CGCACGTCCGTAATTAAC-3′	F12-r9	F: 5′-CGAATCTTAGCCGGAGC-3′ R: 5′- GACACCGTACGAATTTGA-3′
F16-em1	F: 5′- GATCCAGTTACCGGCAC-3′ R: 5′- GACTGCGTACGAATTAAT-3′	F9-em5	F: 5′- GTAGCACAAGCCGGACC-3′ R: 5′-GACTGCGTACGAATTAAC-3′
Me1-r14	F: 5′- TGAGTCCAAACCGGATA-3′ R: 5′-CGCACGTCCGTAATTAAC-3′	F16-r15	F: 5′-GATCCAGTTACCGGCAC-3′ R: 5′-CGCACGTCCGTAATTCCA-3′
Me2-em4	F: 5′- TGAGTCCAAACCGGAGC-3′ R: 5′-GACTGCGTACGAATTTGA-3′	Me2-r9	F: 5′-TGAGTCCAAACCGGAGC-3′ R: 5′-GACACCGTACGAATTTGA-3′
F16-em5	F: 5′-GATCCAGTTACCGGCAC-3′ R: 5′- GACTGCGTACGAATTAAC-3′	F7-r9	F: 5′-GTAGCACAAGCCGGAGC-3′ R: 5′-GACACCGTACGAATTTGA-3′
F16-r14	F: 5′-GATCCAGTTACCGGCAC-3′ R: 5′-CGCACGTCCGTAATTAAC-3′	F9-r9	F: 5′- GTAGCACAAGCCGGACC-3′ R: 5′-GACACCGTACGAATTTGA-3′
Me2-em5	F: 5′-TGAGTCCAAACCGGAGC-3′ R: 5′- GACTGCGTACGAATTAAC-3′		

Table 2. Population polymorphism based on individual of different primer.

Primer	Number of bands	Polymorphic Loci (no.)	Polymorphic Loci (%)
F12-r15	24	19	79.17%
F14-r14	26	24	92.31%
F16-em1	7	7	100.00%
Me1-r14	19	16	84.21%
Me2-em4	8	8	100.00%
F16-em5	3	3	100.00%
F16-r14	30	25	83.33%
Me2-em5	18	5	27.78%
Me4-em4	28	15	53.57%
F12-r9	44	28	63.64%
F9-em5	3	3	100.00%
F16-r15	2	2	100.00%
Me2-r9	12	8	66.67%
F7-r9	20	11	55.00%
F9-r9	8	6	75.00%
Sum	252	180	78.71%
Average	16.8	12	78.68%

Genetic similarity analysis: According to the 0, 1 matrix, we calculated the populations of Nei-Li similarity coefficient, and founded the similarity matrix. The similarity coefficient based on SRAP markers ranged from 0.182 to 0.857 across the accessions with a mean of 0.621 (Table 3). The results showed that there were significant differences among accessions in north region while it seems that, in south region, the similarity coefficient changed slightly and the average coefficient was 0.703. Results showed that 252 polymorphic bands by 15 primers were produced and 180 polymorphic loci were identified ranging from 100 to 2000 bp. Genetic similarity coefficient statistics showed that most of the similarity coefficient was distribution between 0.5 and 0.7, accounting for 52.4% of all, while other coefficient ranging from 0.7 to 0.9, from 0.3 to 0.5 and from 0.1 to 0.3, just occupied 33.3%, 13.0% and 1.3%, respectively. Of the pair wise combinations generated by *Medicago* accessions, Yarkand(2781, No.13) and Ruoqiang (2788, No.20) showed highest similarity index (0.875) and accessions Turpan (2779, No.11) and Burqin (2785, No.17) showed the lowest similarity index.

SRAP derived dendrogram analysis: Nei's genetic distance (Lamboy, 1994) and unweighted pair-group method with arith-metic averages (UPGMA) cluster analysis (Sneath & Sokal, 1973) was carried out and a dendrogram was generated that represented the genetic relationship among 22 accessions from *Medicago* into 5 major clusters. The first major cluster consisted of 3 sub-clusters or groups: four accessions from north of Xinjiang (No. 5, No.7, No.14 and No.15) and one accession from south (No. 1). The second cluster included 11 accessions: four from north (No. 2, No.3, No.4, No.16) and seven from south (No.9, No.12, No.13, No.19, No.20, No.21, and No.22), comprising of most accessions and showing 100% similarity between No.2 and No.4 and No.13 and No.20. The third group consisted of were No.6, No.8, No.10 and No.11 which were all from north, while the fourth and fifth major cluster consisted of accession No.18 and No. 17 alone which appeared to be distinct from all other accessions (Fig. 3). From the group three and four, we can learn that there are some relationship between accessions cluster and geographic distribution and topography. However, there was no significant relationship between group 1 and group 2. This may attribute to complicated background of genetic of alfalfa germplasm. What's more, 14 accessions from north of Xinjiang distributed to four different groups and 8 of them, combining with 8 from south were classified in group 1 and group 2. This reflected that accessions from north were more genetic variation than south; meanwhile it meant there and also had close relationship with ones from south.

Principal component analysis: Based on the genetic similarity coefficient, we mapped the results of the first two main factors by principal component analysis (Fig. 4). All accessions are included in three groups. Group A contained 16 accessions from south and north of Xinjiang; there were 4 accessions which were form north belonging to group B(No.3, No.4, No.5, No.7); the group C has just two accessions: one form south(No.1) and the other from north(No.2). According to principal component analysis (PCA), it is clear that there is some genetic relationship between different *Medicago* accessions. Accession form north was distributed in these three groups and has some closed relationship with others which were form south. This implied that north population's phylogenetic relationship were more complex than south. Genetic distance is quiet significant among *Medicago* accessions from north, while there were cophenetic correlation between north and south populations.

Table 3. Genetic similarity coefficients of *Medicago sativa* accessions from different origins.

Region	No.	Minimum coefficient	Maximum coefficient	Mean
North	14	0.182	0.857	0.564
South	8	0.526	0.857	0.703

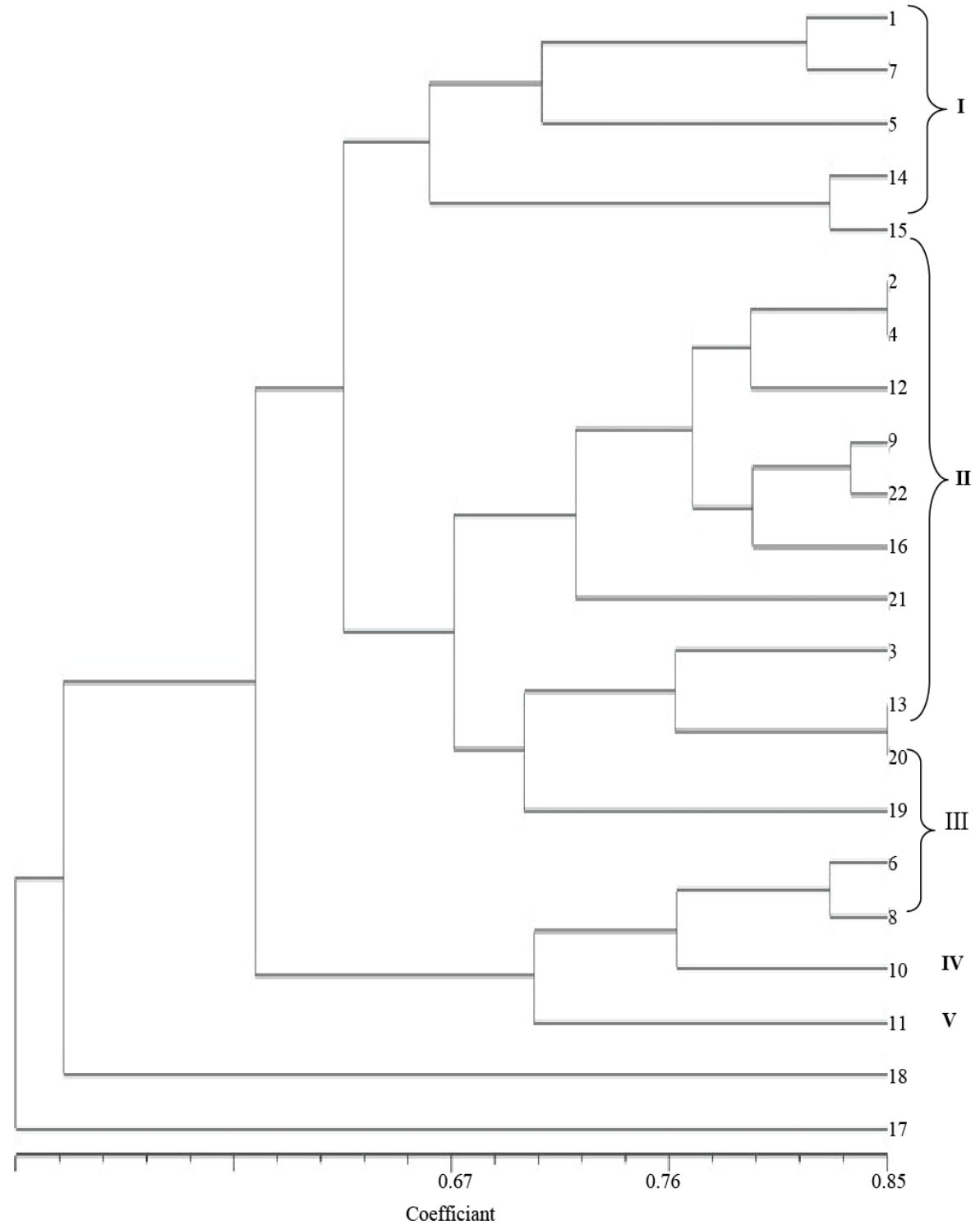

Fig. 3. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on Nei-Li's measures of genetic distances cluster analysis.

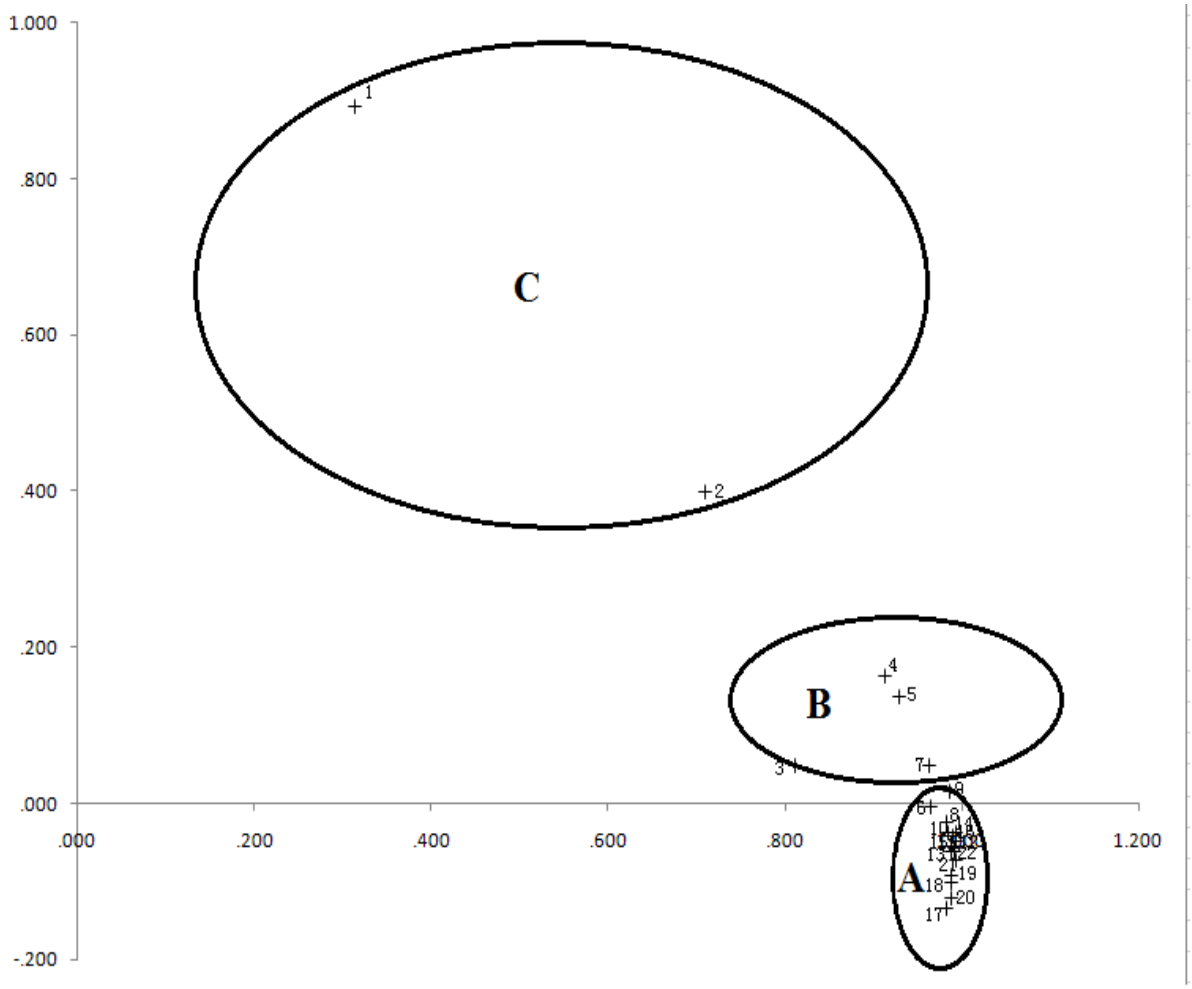

Fig. 4. Principal component analysis based on SRAP makers in *Medicago sativa*.

Discussion

Several molecular markers were used for identification and study of genetic diversity of alfalfa, as AFLP, SSR and RAPD markers are very much used for *Medicago* genus (Segovia-Lerma *et al*., 2003; Li *et al*., 2009; Mohammad *et al*., 2010). Different markers are used to mark the polymorphism. While Volis *et al.,* (2005) argued that a successful management program of *Medicago sativa* cannot be based only markers on RAPDs, ISSRs, SSR, because it might not be correlated to adaptive traits. This study of genetic diversity on 22 accessions of wild *Medicago sativa* from Xinjiang showed that genetic variability was different among accessions observed by SRAP molecular markers. And the average number of polymorphic markers and the percentage of polymorphism were 12 and 78.68%, respectively. Our selected marker collected as high as or higher percentage of polymorphic loci than AFLP (26%, Mohammad *et al*., 2010) and RAPD (67.95%, Noeparvar *et al*., 2008) maker. According to SSR analysis, Li *et al.,* (2009) pointed that to estimate the relationship or genetic diversity of populations more accurately, more DNA loci should be examined in terms of whether SSR markers were related to phenotype or not. Therefore, many primers used in this study generated polymorphic fragments in the accessions of *Medicago*, indicating the usefulness of SRAP analysis to disclose DNA polymorphisms.

Muller *et al*., (2003) argued that the absence of geographical differentiation hinders a more precise location for wild *M. sativa*. Genetic diversity observed in our analysis is not structured according to the geographic origins because the groups obtained in a classification aren't related to topography and climate. Due to the most accessions collected from north where area was extensive, the climate and geographic factors lead to genetic similarity coefficient changed obviously. In south, however, the result showed close genetic proximity with the sympatric wild accessions in genetic diversity. These results indicate that location plays an important role in genetic diversity of *Medicago sativa*. This result is in accordance with other studies analyzed *lucerne* (*Medicago sativa* L.) in south Tunisia (Benabderrahim *et al.*, 2009). Principal component and cluster analysis reveal a considerable variability. Twenty-two accessions are classified into 5 major clusters (Fig. 4) and each cluster almost consisted by individuals form different region. Cluster 2 obtained in dendrogram was formed by 11 accessions: seven from south region and four from north region. Therefore, our study suggested that genetic distance is complicated in different study regions.

Conclusion

The Medicago genus is a good materials for genetic researches and have great potential for breeding and development of forage (Xavier *et al*., 2011). Results suggest that *M. sativa* shows genetic variability among accessions in Xinjiang region. And, our results also showed that there is closed relationship between accessions from different geographical regions while low similarity appears within the same region. It suggests that the observed genetic similarity is mainly caused by climate and tropology. Additionally, our study proved that SRAP marker can be used as a successful management program to acquire genetic loci. In generally, wild *Medicago* has abundant genetic diversity in Xinjiang region and these results are beneficial to protect alfalfa germplasm resources for developing local husbandry.

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