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Full Length Article

Genetic Diversity in the Worldwide Alfalfa Germplasm Assessed through SSR Markers

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Abstract

It was an effective measure for speeding up the process of alfalfa breeding to dissect genetic diversity and population structure of the worldwide alfalfa germplasm. A total of 242 worldwide alfalfa (*Medicago sativa* L.) accessions were genotyped using 102 pairs of SSR primers evenly distributed on 8 chromosomes of alfalfa genome. The results showed that 471 alleles were generated. One marker was not polymorphic, 13 markers showed a homozygous genotype and 89 markers showed a heterozygous genotype in the worldwide germplasm. The total *PIC*, N_a, N_e, H_0 and H_j values were 0.4627, 4.1324, 2.4671, 0.9426, and 0.5147, respectively. These values were high in the perennial wild population and were lowest in the annual population. A lower genetic diversity was detected in alfalfa annual population than others. The five natural populations of cultivation types were classified into two clusters, corresponding to perennial and annual populations. The principal coordinates analysis (PCoA) and unweighted group method with arithmetic mean (UPGMA) tree revealed that 242 accessions could be gathered into three groups. The same natural population gathered together but did not completely cluster into the same genetic group. The analysis of molecular variance analysis (AMOVA) showed that the total genetic differentiation (*Fst*) and gene flow (*Nm*) among all populations were 0.08 and 2.8659, respectively. The *Fst* and *Nm* among perennial populations were 0.03 and 7.5995, respectively. The molecular variation of within populations and among populations accounted for 94.94 and 5.06% in all populations. 96.71 and 3.29% of molecular variation were occurred within and among in perennial populations. These results showed that the wild perennial alfalfa population has higher genetic diversity than the others. There was a larger genetic barrier and less gene exchange between the annual and perennial populations. © 2019 Friends Science Publishers

Keywords: Alfalfa; SSR markers; Germplasm assessed; Genetic diversity

Introduction

Alfalfa is widely cultivated all over the world, about 80 million hectares worldwide. It has the advantages of high biomass, high protein content, nitrogen-fixing ability, suitable feeding value and favorable environment *etc*. (McCord *et al*., 2014). Currently, breeders are breeding varieties to adapt to different ecotypes and for the further expansion of the alfalfa planting region. The speed of the breeding process will be determined by the level of mastering the richness of germplasm and the study depth of germplasms' genetic laws. It is better to guide the breeding varieties and accelerate the process of breeding by deathly dissect the genetic law and the evolution mechanism of mastering germplasm.

Alfalfa has evolved into different taxa, which include both diploid (2n= 2x = 16) and tetraploid (2n= 4x = 32) varieties with only a weak hybridization barrier at the same and across ploidal levels. The principal diploid taxa include *M. sativa* subspp. *caerulea*, *M. sativa* subspp. *falcata* (L.), and *M. sativa* subspp. *glomerata* (Balb.). Other species were caused by polyploidization and hybridization. Diploid *M. sativa* subspp. × *hemicycla* is a putative natural diploid hybrid between *caerulea* and diploid *falcata*. The tetraploid taxa include *Medicago sativa* subspp. *sativa* (the direct analogue of diploid subspp. *caerulea*), "tetraploid *falcata*", "tetraploid *glomerata*", *M. sativa* subspp. × *varia* and *M. sativa* subspp. ×*tunetana* (Havananda *et al*., 2011). However, the genetic validity classification of subspecies has not been confirmed in previous studies.

Alfalfa is generally considered to have originated from Transcaucasia, Turkmenistan, Iran, and Asia Minor. Since then, it has diversified into the Mediterranean area and has spread and evolved around the world (Barnes *et al*., 1977; Touil *et al*., 2008). Previous studies have shown that *falcata* and *caerulea* accessions were clearly distinct (Şakiroğlu *et al.,* 2010) and *Falcat*a and *sativa* were similarly distinct (Havananda *et al*., 2010, 2011). Relationships and genetic diversity among these germplasms have been previously characterized using other molecular markers (Vandemark *et*

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al., 2006; Touil *et al.*, 2008). Population structures of cultivated tetraploid alfalfa have also been estimated (Qiang *et al.*, 2015).

In alfalfa, many markers have been broadly applied for various studies, such as biochemical markers applied for resistance study (Maghsoodi *et al.*, 2017), different molecular markers applied for population structure and diversity (Vandemark *et al.*, 2006; Touil *et al.*, 2008; Bhandari *et al.*, 2011), genetic QTL mapping (Julier *et al.,* 2003; Choi *et al.,* 2004), and association mapping (Wang *et al.,* 2016) *etc*. The markers of SSR and SNP have many advantages, therefore, they were more widely utilized these studies (Robins *et al.,* 2007; Li *et al.,* 2014).

However, these studies were limited distinctions, genetic diversity among subspecies or individual only using a limited range of alfalfa accessions (Li and Brmmer*,* 2012), such as only using wild germplasm (Greene *et al.,* 2008) or core germplasm (Vandemark *et al.,* 2006). A comprehensive study involving a worldwide collection of diploid and tetraploid alfalfa germplasm is still needed to dissect the population structure and genetic diversity, as well as the relationship between genetic variation and geographical regions in worldwide alfalfa, for its effective selection of parents for breeding and to speed up the breeding process. Therefore, we selected a broad range of alfalfa germplasms worldwide, including America, Canada, Australia, China, and Europe, in order to estimate the genetic diversity, population structure and evolutionary relationship of different taxa of alfalfa using SSR markers.

Materials and Methods

Plant Materials

A total of 242 alfalfa accessions worldwide were used in the study. Thirty accessions were collected from being popularized alfalfa varieties in China. The remaining 212 accessions were obtained from the U.S.D.A. National Plant Germplasm System (NPGS). There were nine annual alfalfa accessions, including one wild, one cultivar, and seven with uncertain improvement status. Among the 233 perennial alfalfa accessions (Table S1), there were wild (24), landrace (42), cultivar (76), and uncertain accessions (91).

DNA Extraction and SSR Analysis

For each accession, genomic DNA was extracted from three independent plant fresh leaves, following the CTAB method with minor modifications (Saghai-Maroof *et al.,* 1984). According to the sequence published on Sledge *et al.* (2005), 102 pairs of SSR primers (Table S2) distributed evenly on the genetic map were selected for genotyping the242 accessions. All SSR reactions were performed in 10 μL containing approximately 50 ng of genomic DNA, 1 μL 10× PCR buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.0, 0.1% gelatin), 0.60 μmol/L of both forward and reverse primers, 0.2 mmol/L dNTPs and 1 U Taq polymerase in double distilled water. The PCR program was used: a pre-denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 50 s, extension at 72ºC for 50 s; and a final extension at 72ºC for 10 min before cooling to 12ºC. PCR products were visualized after electrophoresis on an 8% polyacrylamide gel followed by silver staining (Sanguinetti *et al.,* 1994).

Data Analyses

The genotypes of 242 accessions were detected by 102 pairs SSR markers which distributed evenly on 8 chromosomes of alfalfa. The molecular weight of the main band of each marker was read by software of Quantity one. The total number of alleles (*TNB*), number of polymorphic loci (*NPB*), percentage of polymorphic loci (*PPB*) were calculated using Excel 2017 software. The polymorphism information content (*PIC*), observed number of alleles per locus (*Na*), effective number of alleles per locus (*Ne*), Shannon information index (I), and Nei's genetic diversity (*Hj*) were calculated using Popgene32 (Yeh *et al.*, 1999).

The *Nei's* genetic distance (*GD*), genetic identity (*GI*), Gene flow (*Nm*), genetic differentiation (*Fst*), the Shannon differentiation coefficient (G'st), and the genetic correlation among different natural populations were analyzed using Popgene32.

The genetic similarity coefficient among different accessions was calculated and a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) by PowerMarker3.25 software (Liu and Muse, 2005). The principal coordinate analysis (*PCoA*) was analyzed using GenAlEx6.5 software (Peakall and Smouse, 2012). Analysis of molecular variance (AMOVA) was conducted using GenAlEx 6.5 software.

Results

Results of Primer Amplification

The amplification effect of these primers was better. These primers rarely amplified bands in accessions. There was a minimum of six pairs of primers (Chr.6) and a maximum of 19 pairs (Chr.8) on each chromosome with an average of 12.75 pairs on each chromosome. The 102 markers generated 471 alleles that corresponded to an average of 4.62 alleles per marker. The alleles of each marker ranged from 1 to 8. Among these markers, only marker AW127 was not polymorphic in the total population. Thereinto, 13 markers detected homozygous genotype on all resources, and 89 of markers had heterozygous genotype in the worldwide alfalfa germplasms.

Genetic Diversity Analysis Within Natural Populations

The genetic diversities of five natural populations are listed in Table 1. The number of polymorphic loci (*NPB*) ranged

Table 1: Summary of genetic diversity in five life from natural populations of alfalfa

Populations	No. of sample	NPL	PPL%	PIC	Na	Ne	H_0	H_j
PL	42	95	93.14	0.4254	3.3235	2.2614	0.8473	0.4805
PU	100	97	95.10	0.4499	3.7696	2.4010	0.9104	0.5019
PW	24	96	94.12	0.4734	3.5049	2.4973	0.9483	0.5259
PC	67	98	96.08	0.4353	3.5490	2.3296	0.8752	0.4892
Annual	9	90	88.24	0.3882	2.7598	2.0487	0.7562	0.4415
Total	242	99	97.06	0.4627	4.1324	2.4671	0.9426	0.5147

NPL, number of polymorphic loci; *PPL*, Percentage of polymorphic loci; *PIC*, Polymorphism information content; *Na*, Observed number of alleles per locus; *Ne*, Effective number of alleles per locus; H_0, Shannon diversity index; *Hj*, *Nei's* genetic diversity

Table 2: Nei's genetic distance and genetic identity (1972) between life from populations

Populations	Perennial landrace	Perennial uncertain	Perennial wild	Perennial cultivar	Annual
PL	****	0.9706	0.9483	0.9574	0.7742
PU	0.0298	****	0.9502	0.9642	0.8198
PW	0.0531	0.0510	****	0.9462	0.8103
PC	0.0436	0.0365	0.0553	****	0.8149
Annual	0.2559	0.1987	0.2104	0.2047	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

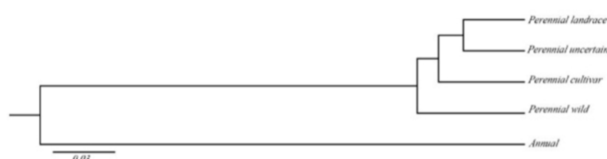

Fig. 1: Natural population dendrogram of worldwide alfalfa based on *Nei's* genetic distance

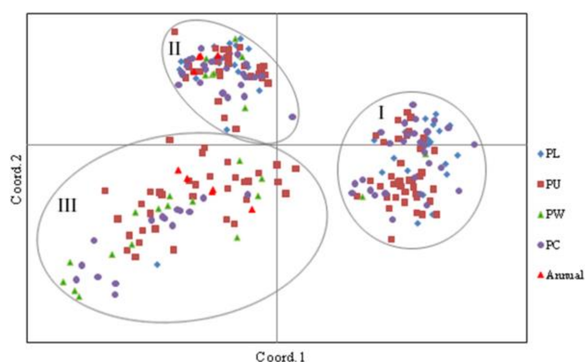

Fig. 2: Principal coordinates analysis (PcoA) from five natural populations based on the genetic distance matrix
Population's code: Perennial landrace (PL), Perennial uncertain (PU), Perennial wild (PW), Perennial cultivar (PC), and Annual. The same below. The five populations were represented by different colors and shapes

from 90 (Annual population) to 98 (perennial cultivar's population), and the total *NPL* was 99. The total percentage of polymorphic loci (*PPL*), polymorphism information content (*PIC*), observed number of alleles per locus (*Na*), effective number of alleles per locus (*Ne*), Shannon diversity index (H_0) and *Nei's* genetic diversity (H_j) were 97.06, 0.4627, 4.1324, 2.4671, 0.9426, and 0.5147%, respectively. The *PPL* was highest in the perennial cultivar population, the *Na* was highest perennial uncertain population, and the *PIC*, N_e, H_0, and H_j were the highest in perennial wild population. These values were lowest in the annual population.

Genetic Distance and Structure of among Natural Populations

The genetic identities and distances were listed in Table 2 among natural populations. The genetic identity was relatively high (GI ≥ 0.9483) and the genetic distance was relatively low (GD ≤ 0.0531) among perennial natural populations. The genetic distance was relatively high between perennial and annual natural populations, varying from 0.1987 (Perennial uncertain *vs.* Annual) to 0.2559 (Perennial landrace *vs.* Annual).

To study the genetic structure of different life forms of world alfalfa, a dendrogram was generated by Popgene32 and MEGA4 software with the UPGMA method (Fig. 1). The results showed that the 5 natural populations gathered into two clusters. Cluster I contained 4 perennial populations (landrace, uncertain, cultivar, wild), whereas Cluster II only contained 1 annual population. The genetic distance between the landrace and uncertain is the nearest, followed by between them and cultivar, relatively far away is between wild and others in perennial populations.

Further analysis showed that genetic differentiation among natural populations (*Fst*) of world alfalfa was higher than perennial natural populations. The total *Fst* of the whole group was 0.08 (range: 0.00 to 0.35). The gene flow (*Nm*) of the whole group varied from 0.4621 to 34.6281 in allele loci, and the total *Nm* value was 2.8659. However, the total *Fst* of perennial natural populations was only 0.03 (range: 0.00 to 0.14) and the gene flow (*Nm* = 7.5995) was high (range: 1.541 to 56.970) among perennial natural populations (Table 3).

The analysis of molecular variance (AMOVA) showed that the molecular variation of within populations accounted for 94.94% and among populations only accounted for 5.06% in all total population. 96.71% of the genetic differentiation was attributable to variability within populations, and 3.29% occurred among populations in perennial populations (Table 3).

Analysis of the Genetic Structure of Genetic Resources

Principal coordinates analysis (PCoA) showed that the first, second and three principal components explained 8.08, 7.05 and 4.86% of the total genetic variation, respectively. These alfalfa accessions were basically divided into three groups. Perennial landrace accessions were divided into group-I and group-II. Three groups contained perennial uncertain and cultivar resources. Perennial wild and annual accessions

Table 3: Analysis of molecular variance for different populations

Group	Source Of Variation	Degree Of Freedom	Mean Square	Variance Components	Percentage Of Variation (%)	*Nm*	*Fst* (%)
All pops.	Among pops.	4	11471837.55	185608.93	5.06	2.8659	8.02
	Within pops	237	3484901.29	3484901.29	94.94		
Perennial pops.	Among pops.	3	23287.35	280.50	3.29%	7.5995	3.18
	Within pops.	233	8255.18	8255.18	96.71%		

Table 4: Distribution of genetic resources of five natural populations in three genetic groups

	PL	PU	PW	PC	Annual	Total
Group-Ⅰ	0	9	8	1	7	25
Group-Ⅱ	22	32	10	19	0	83
Group-Ⅲ	20	59	6	47	2	134
Total	42	100	24	67	9	

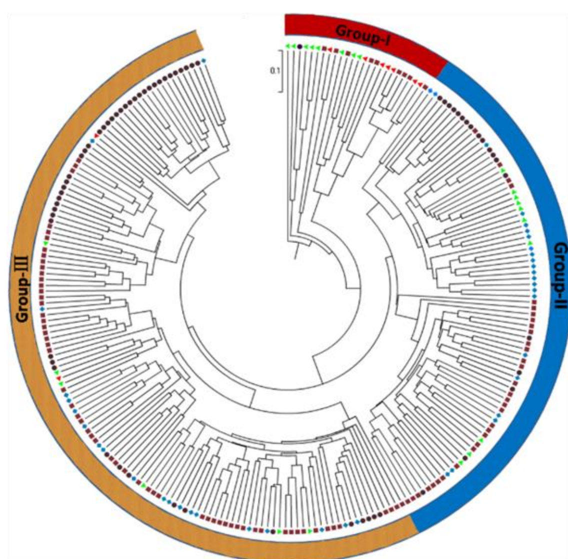

Fig. 3: Dendrogram of 242 alfalfa accessions (UPGMA) using data of SSR markers

were basically divided into group-II and group-III (Fig. 2).

The phylogenetic relationships among 242 alfalfa accessions were analyzed using 102 SSR markers. The dendrogram was constructed using the UPGMA method (Fig. 3, Table 4). The genetic similarity was highest among accessions of group-III, and the smallest was among accessions of group-I. The same natural population gathered together, but did not completely cluster into the same genetic group from the distribution condition. The genetic diversity of perennial cultivars is the smallest and most of them (70.15%) were clustered into group-III. The all accessions of PL and the vast majority of PU were clustered into group-II and group-III, the genetic diversities of them are smaller. The distribution of PW's accessions was similar in the three groups, the genetic diversity of some of accessions was large and others were small in the natural population. The majority of accessions of the annual population (77.78%) were clustered into group-I the diversity of them is large.

Discussion

Genetic stock represents all kinds of genes evolved in the process of evolution. It is the material basis of crop breeding, and also the basis of the research of crop origin, evolution, classification, and inheritance. Abundant genetic resources provide alternative variation for breeding. Selection, genetic drift and gene flow act on the alleles present in different populations to cause variation in the diversity in them. The selection can be natural or artificial (Rao and Hodgkin, 2002). A better understanding of genetic diversity can make better utilization of related resources.

The genetic diversity of worldwide collection of 242 alfalfa accessions was dissected using 102 SSR markers. The genetic diversity of alfalfa germplasm in the study was lower than previous studies (Flajoulot *et al.,* 2005; Li *et al.,* 2014; Qiang *et al.,* 2015) as reflected in average allele number per locus (4.62), *Na* (4.1324), *Ne* (2.4671) and *PIC* (0.4627). The PIC value of five natural populations ranged from 0.3882 of annual to 0.4734 of perennial wild. This experiment revealed the population of perennial wild had relatively higher genetic diversity than others, and the population of annual had lower. The selection of landraces and cultivars resulted in diversity loss and a bottleneck. The previous study on nuclear gene sequences also showed that the bottleneck existed in the domestication of at least some alfalfa populations (Muller *et al.,* 2006). There is less gene exchange between the annual and perennial alfalfa.

The genetic identity was high among perennial natural populations. However, the genetic identity was low between annual and perennial populations. These indicated that there is more gene flow among perennial natural populations and there is less gene exchange between the perennial and annual populations.

Alfalfa is autopolyploidy, it mostly involves genome duplication. An autopolyploid has been from a similar genetic background or possibly from a single progenitor genotype (Havananda *et al.,* 2011). Perennial alfalfa is mostly autotetraploid, and annual is diploid. There are few genetic exchanges between the annual and perennial alfalfa in the study, or the breeding of hybrid progeny is more difficult. The previous study showed that the *falcata* and *caerulea* accessions are clearly distinct, with hybrids falling in between the separate parental groups (Şakiroğlu *et al.,* 2010).

There is a certain genetic differentiation between within and among populations, the genetic diversity of perennial wild population is the highest (*Nei* = 0.5259), the

annual population is the lowest (*Nei* = 0.4415). The AMOVA also showed that there was little variation (3.29%) among the perennial populations and a relatively large variation (5.06%) between the annual and perennial populations. The gene differentiation (*Fst*) is smaller among perennial populations than between annual and perennial populations. All these indicated that there are more genetic exchanges among perennial populations than between perennial and annual populations.

The cultivated population has fewer alleles, it is more surprising that the number remains very high, particularly some materials derived from only one breeding program representing years of selective breeding. However, abundant allelic variation exists in the wild germplasm and, presumably, some of them will be useful for breeding, adding desirable alleles for key traits (Şakiroğlu *et al.,* 2010). Dissection of the genetic relationship of the different accessions may guide the selection of alfalfa parents. Population structure analysis enables to identify the appropriate population and exclude the effect of consanguinity relationship for association mapping (Zhang *et al.,* 2010). All accessions of different natural populations were divided into three genetic populations (Fig. 2, 3 and Table 4), which define the genetic relationship among the different accessions to provide theoretical guidance for parent selection in alfalfa breeding.

Conclusion

The*PIC*, N_a,N_e, H_0 and H_j values were high in the perennial wild population and were lowest in annual population. A lower genetic diversity was detected in alfalfa annual population than others. The five natural populations were classified into two clusters, corresponding to perennial and annual populations. 242 accessions were clustered into three groups. The same natural population gathered together but did not completely cluster into the same genetic group. The total genetic differentiation (*Fst*) among all populations was higher than among perennial populations. The molecular variation of among populations accounted for lower in all populations than in perennial populations. These results showed that the wild alfalfa population has higher genetic diversity than the others. There was a larger genetic barrier and less gene exchange between the annual and perennial populations.

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