



Genetic Diversity Study of Some Iranian Alfalfa Genotypes Based on Seed Storage Proteins Patterns

Mehdi Kakaie¹ · Soolmaz Ahmadian¹

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Abstract

Alfalfa (*Medicago sativa*), known as green gold, is one of the best-known forage plants in the world. Alfalfa is an autotetraploid allogamous plant with a large genetic diversity among its cultivars. This diversity is due to self-incompatibility, a high rate of allogamy, and open pollination. This study aimed to investigate the biodiversity of grain storage proteins of *M. sativa* using gel electrophoresis. For this purpose, seed proteins of 12 Iranian genotypes including seeds collected from Lenjan of Isfahan, Dashte Qazvin, Asadabad, Qasr Shirin, Taleghan Qazvin, Malayer, Isfahan, Saqez, Gonbad Kavous, Kamyaran, Shiraz, and Najafabad of Isfahan were studied and a total of 22 strips were identified. Then, the strips were used to estimate the genetic distance of alfalfa genotypes. The results of the study based on the presence and absence of specific protein bands in the seed protein pattern showed that genotypes 1 (Collection from Isfahan-Lenjan) and 12 (Collection from Najafabad-Isfahan) have the most variation. A similarity matrix was calculated and cluster analysis was performed based on the Jaccard similarity coefficient by the UPGMA method. The similarity coefficients ranged from 0.3 to 1. In general, classification based on cluster analysis showed a similar trend to the differences observed in gel electrophoresis. The total protein of population number 12 (Najafabad population of Isfahan) had the highest amount among the studied genotypes. The highest and lowest number of bands were related to genotype number 12 (population of Najafabad, Isfahan) and genotype number 11 (population collected from Shiraz), respectively. Therefore, the results showed that the protein pattern of alfalfa seeds based on the presence and absence of protein bands can determine genetic diversity. These findings can be used in future research in the field of proteomics and breeding studies in future.

Keywords Total soluble protein · SDS–PAGE · Genetic distance · Dendrogram via UPGMA

1 Introduction

Alfalfa (*Medicago sativa* L.) is one of the high-quality forage crops in the world. This plant has special nutrients including proteins, minerals, vitamins, and some secondary metabolites such as saponin, which is superior to other forage plants (Kakaie and Mazahery-Laghab 2014). Alfalfa has a large variability in the genome due to its autotetraploid genetic structure ($2n = 4x = 32$) and cross-pollination. The existence of high variability makes it difficult

to identify different varieties by agronomic and morphological traits (Corts and Martinez 2000). On the other hand, seed storage proteins are powerful tools for studying genetic diversity (Kakaie and Kahrizi 2011). Therefore, the use of protein markers such as seed storage proteins in breeding programs is highly considered (Javaid et al. 2004).

Strong proteins are significantly stable, despite, high polymorphisms (Valizadeh 2001). Environmental factors have little effect on ripe seed proteins and how they are inherited is codominant. The ability to distinguish between two species is important in plant breeding as well as cultivar registration, and this issue is very important when deciding on fertilization between cultivars. Due to the allogamy and tetraploid of alfalfa, it seems that there is a lot of genetic diversity within alfalfa populations. Some studies have shown that the greater the genetic distance

✉ Mehdi Kakaie
m.kakaie@pnu.ac.ir; mehdikakaie37@gmail.com
Soolmaz Ahmadian
soolmazahmadian@yahoo.com

¹ Department of Agriculture (Plant Breeding), Payame Noor University, Tehran, Iran

between individuals in the population, the higher the forage yield (Kidwell et al. 1994).

The study of seed storage proteins using gel electrophoresis (SDS–PAGE) is still a simple, fast, cheap, and common way to evaluate the differences among protein patterns of cultivars and their genetic diversity. Genetic diversity is not only the basis of breeding studies to identify the plant species with better adaptation and higher tolerance to biotic and abiotic stresses, but also it is necessary to determine the amount of genetic diversity between and within alfalfa species (Golan 2013). In a study, Fareghi et al. (2007) of the electrophoretic pattern (SDS–PAGE) classified some perennial alfalfa populations into three distinct groups by cluster analysis and suggested populations to create different species. In Stoyanova and Boller (2010) studies, they used seed protein electrophoresis to study and classify genetic diversity within *Meadow Fescue* genotypes and found these techniques useful in breeding and seed industry applications. Therefore, the genetic purity of seeds can be determined by using the similarity of protein bands in a short time and without performing agricultural operations.

Therefore, this study aimed to investigate the extent of diversity and clearly to know of genetic distance using protein markers that can be used to identify suitable parents for hybridization and hybrid development programs.

2 Materials and Methods

In this study, the seeds of 12 genotypes (table) were studied. For this purpose, first, the seeds were collected, frozen in liquid nitrogen, and then ground into a powder with liquid nitrogen. The samples were placed in 2-ml microtubes. Then after filling with protein extraction buffer, they were centrifuged for 20 min at 4 °C with 13,000 g. After the supernatant was transferred to another centrifuge tube, filled with TCA (Tricolor Acetic Acid)—Aceton solution and left in the freezer for 1 h. It was then centrifuged at 4 °C at 13,000 g for 20 min. After the protein precipitated, the supernatant was discarded and washed with acetone and then mixed with a lysis buffer of 150 µl according to Table 1 (Xi et al. 2006 with amount change).

Table 1 Lysis Buffer used to extract protein

Urea	7 M	1.26126 g
Thiourea	2 M	1.59831 g
PMSF	1 mM	0.03 µl
Tris-base	20 mM	0.007284 g
DD H2O		To 3 ml

Finally, it was shaken for 1 h and read by Bradford (1976) method, in the range of 595 nm. SDS–PAGE analysis was performed using 12.5% separating gel by Laemmli (1970) method with some modifications. Protein separation time was about 2 h. And Protein bands were then stained with Coomassie Brilliant Blue (CBB) R-250. Finally, the position of each strip on the gel was determined as codes one and zero, which indicate the presence and absence of the relevant strip, respectively. To obtain cluster analysis, first, the matrix of genetic distances of the data was determined by NTSYS Pc 2.2 software, then the dendrogram was drawn by the UPGMA method and the kinship relationships of the studied genotypes of alfalfa were evaluated. The original coordinate analysis was also plotted by NTSYS software.

3 Results and Discussion

In this study, the results of protein electrophoresis by the SDS–PAGE method on the studied genotypes, a large number of bands on polyacrylamide gel were detected and the position of each band was determined by determining their relative movement distance. In total, 22 bands with different mobility were identified on the gel (Fig. 1). In terms of the number of protein bands, light molecular weight proteins produced more protein bands and heavy molecular weight proteins produced fewer bands. Since protein bands are the final product of genes, they represent a pattern of genotypes that can be used to group genotypes. In another research, the genetic variability of annual *Medicago* species was examined with SDS–PAGE, and in that study, researchers showed low polymorphism, but they revealed that the number of bands was relatively high (Fyad-Lameche et al. 1996). Another researcher in the study seed storage protein soybean cultivar showed that there were significant differences among genotypes for seed protein content (Arefrad et al. 2014). Also, SDS–PAGE was used in a study to detect the genetic diversity of perennial Lucerne (*Medicago sativa* L.) (Fareghi et al. 2007).

The overall results of a study indicated that SDS–PAGE was a useful tool for genetic diversity analysis and laid a solid foundation for future *faba bean* breeding. In that study, the genetic diversity and relationships of 101 *faba bean* (*Vicia faba* L.), landraces, and varieties from different provinces of China and abroad were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (Hou et al. 2015). In the present study, the dendrogram obtained from the analysis of electrophoretic data by the UPGMA method divided the genotypes into four groups (Fig. 2). The first group includes genotypes No. 1, 8, and 11, the second group includes genotypes No. 2 and

Fig. 1 *Medicago sativa* protein banding patterns using SDS–PAGE (the genotype names and numbers are according to Table 2)

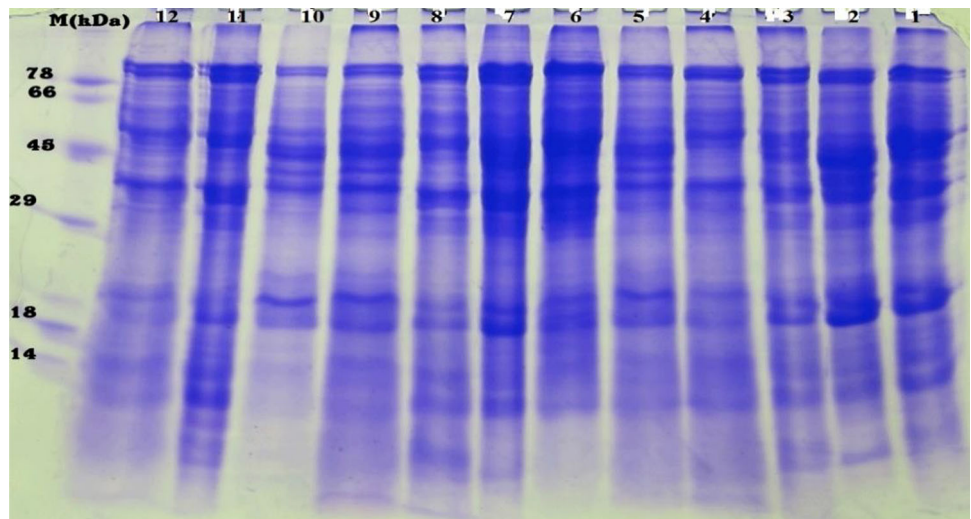


Table 2 The *Medicago sativa* genotypes that have been used in the current study

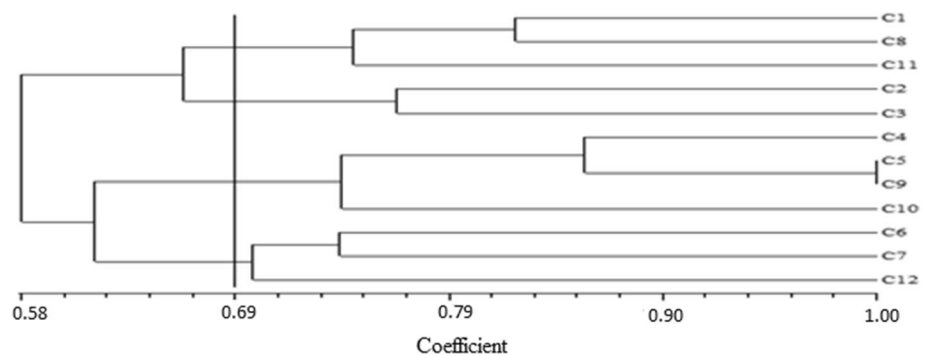
No.	Gathering location	No.	Gathering location	No.	Gathering location	No.	Gathering location
1	Collection from Isfahan-Lenjan (In the Center of Iran)	4	Collection from Qasr Shirin (West of Iran)	7	Collection from Isfahan (In the Center of Iran)	10	Collection from Kamyaran Yozider (West of Iran)
2	Collection of Dashte Qazvin (In the Center of Iran)	5	Collection of Taleghan Qazvin (In the center of Iran)	8	Collection from Saqqez (Northwest of Iran)	11	Collection from Shiraz (South of Iran)
3	Collection from Qasem Abad Asadabad village (West of Iran)	6	Collection from Malayer (West of Iran)	9	Collection of Gonbad Kavous (North of Iran)	12	Collection from Najafabad-Isfahan (In the Center of Iran)

3, the third group includes genotypes No. 4, 5, 9, and 10, and the fourth group includes genotypes No. 6, 7 and 12. Therefore, in the electrophoresis study of *M. Sativa* genotypes, four protein groups can be introduced. Also, the maximum distance was observed between the two genotypes No. 1 and 12, which can be used for breeding purposes in crossbreeding programs.

In another study, seven landraces of cowpea (*Vigna unguiculata* L.) were assessed for genetic variability in total proteins by SDS–PAGE and showed that seed proteins

provide valuable information regarding the variability among landraces and they also revealed that information could be utilized for breeding purposes in the enhancement of protein quality and quantity in grain legumes (Alghamdi et al. 2019). This study aimed to evaluate the genetic diversity and geographical groups of some Iranian alfalfa genotypes to use in future breeding studies. Seed storage proteins are very stable despite being highly polymorphic, so the electrophoretic pattern of seed storage proteins alone or with other markers would be a good criterion for

Fig. 2 Dendrogram for *Medicago sativa* population based on SDS–PAGE markers via UPGMA method



identifying plant populations (Lawrence et al. 1987). Zivkovic et al. (2012) assessed genetic diversity among alfalfa (*Medicago sativa* L.) genotypes by morphometry, seed storage proteins, and RAPD analysis. And they showed these methods provided a rapid way to discriminate between genotypes. Random amplified polymorphic DNA (RAPD) was used in another study to evaluate the genetic variability of 7 alfalfa cultivars in Northwest China which indicated that a high diversity existed in those cultivars (Wang et al. 2011). The Genetic Diversity of Alfalfa Cultivars was also assessed by Microsatellite Markers (Shamustakimova and Klimenko 2019). Hamouda (2019) reported that SDS protein is one of the important tools for genetic analysis for *Silybum marianum* and recommended to give accurate results. Genetic Diversity among Selected *Medicago sativa* cultivars using inter-retrotransposon-amplified polymorphism, chloroplast DNA barcodes, and morpho-agronomic trait analyses was assessed and opened a new possibility of producing *M. sativa* lines that have high forage quality, productivity, and resistance to biotic and abiotic stress conditions. For these objectives, the collection and characterization of genetic resources are required for the development of new cultivars (Badr et al. 2020) Therefore initial screening can be used by SDS–PAGE.

In a study Darvishi-Zaidabadi et al. (2000) on different alfalfa cultivars by seed protein storage electrophoresis method reported many similar bands among the studied populations, which is in line with the results of the present study. The cophenetic correlation coefficient computed for the goodness of fit of the dendrograms of this study with qualitative data is 0.71, which indicates the acceptable fit of the dendrogram with the qualitative data. The use of dendrograms from NTSYS software and principal component analysis based on molecular data has been used by Ismaili et al. (2013) in some thyme genotypes which proves the efficiency of grouping genotypes based on molecular qualitative data. Hamouda (2019) showed that the PCA biplot classification of the studied alfalfa cultivars

confirmed the affinity between the two Egyptian cultivars. Nowadays, awareness of genetic diversity and the management of genetic resources are considered important components of breeding projects. Because most of the genes responsible for resistance to diseases, pests, environmental stresses, and genes responsible for product quality are found in diversity centers. Therefore, it is necessary to pay attention to the geographical areas of the country in collecting alfalfa germplasm. Due to this issue, breeders can use the genetic diversity of each plant more efficiently than exploit genetic resources and directly collect the required hereditary reserves (Haidari et al. 2014).

3.1 Results of Intraspecific Diversity in *Medicago sativa* by principal component analysis (PCA)

Figure 3 shows the diagram obtained from the analysis of the main axes using the presence and absence of protein bands (based on alfalfa seed proteins of the studied alfalfa genotypes) on the coordinate axis. These results are in line with clustering resulting from cluster analysis. Genotypes 2 and 3 are adjacent to each other on biplot space. Also, genotypes 1, 8, and 11 are close to each other. Genotypes 4, 5, 9, and 10 are in the third cluster. Finally, the other genotypes, namely genotypes 6, 7, and 12, are located in the fourth cluster of cluster analysis and close to each other, which is shown in Fig. 3.

3.2 Total Soluble Protein Content

Figure 4 shows a diagram of total protein in the studied alfalfa genotypes. In terms of protein content, there are differences between the studied genotypes, which due to the importance of protein content (quantitatively) can be considered in the study of alfalfa in breeding activities. The highest amount of protein was related to genotype 12 and genotype 6, respectively, and the lowest amount was related to genotype 11.

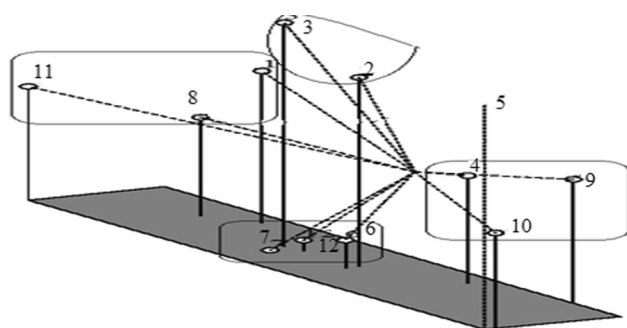


Fig. 3 Analysis chart of the main topics on alfalfa seed proteins studied alfalfa genotypes (numbers 1 to 12 numbers by table are alfalfa genotypes)

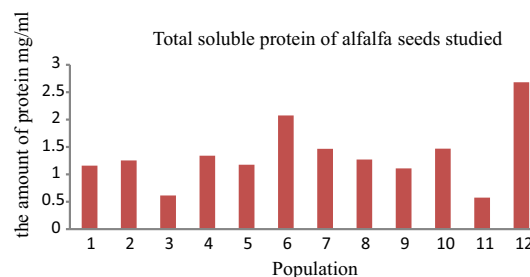


Fig. 4 Chart of total protein, alfalfa genotypes studied (numbers 1 to 12 numbers alfalfa genotypes according to table)

Table 3 Jaccard's similarity matrix based on protein markers studied alfalfa genotypes (numbers 1 to 12 numbers alfalfa genotypes according to table)

	1	2	3	4	5	6	7	8	9	10	11	12
1	1											
2	0.736	1										
3	0.812	0.764	1									
4	0.647	0.611	0.666	1								
5	0.666	0.722	0.687	0.857	1							
6	0.619	0.666	0.476	0.578	0.684	1						
7	0.631	0.523	0.473	0.588	0.611	0.736	1					
8	0.823	0.6	0.647	0.687	0.611	0.571	0.666	1				
9	0.666	0.722	0.687	0.857	1	0.684	0.611	0.611	1			
10	0.5	0.555	0.5	0.642	0.785	0.526	0.529	0.444	0.785	1		
11	0.722	0.523	0.647	0.5	0.45	0.5	0.578	0.764	0.450	0.3	1	
12	0.666	0.476	0.5	0.733	0.647	0.684	0.705	0.705	0.647	0.562	0.611	1

3.3 The Migration Pattern of the Bands

Table 3 shows the Jaccard similarity matrix based on seed protein markers of the studied alfalfa genotypes. The calculation of similarity coefficients by the Jacquard method showed a range from 0.3 to 1. Genotypes 9 and 5 have the most similarity (1) and genotypes 11 and 10 have the least similarity (0.3). In a study Musavi et al. (2010), to investigate the diversity of glutenin subunits in *Aegilops tauschii* genotypes, showed that the similarity coefficients by the Jacquard method range from 0.42 to 1. The existence of differences and diversity in protein bands in the studied genotypes indicates the existence of intraspecific diversity and shows that SDS–PAGE is a suitable biochemical method for classifying alfalfa genotypes. The highest amount of total soluble protein was related to the genotype collected from Najafabad, Isfahan. The results of the study of kinship relationships based on the presence or absence of protein bands showed that genotypes 1 and 12 were the most different from each other based on cluster analysis dendrograms which can be used in cross-breeding experiments of breeding projects.

4 Conclusion

Seed storage proteins profiling provides aid in the identification and characterization of diversity among the landraces and prevailing their phylogenetic relationships (Nisar et al. 2007). The divergence observed in alfalfa species should be further confirmed using molecular tools. Protein profiling is an efficient method for studying genetic diversity, coupling biochemical methods with molecular approaches would help plant taxonomists to understand the variance among plants in further depth. Also, by examining the field traits of these genotypes, it is possible to match the agronomic traits with the molecular data. It is also

suggested that molecular data from DNA markers be examined in the future, of course, with more genotypes.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent This article does not contain any studies with human participants.

References

- Alghamdi SS, Khan MA, Migdadi HM, El-Harty EH, Afzal M, Farooq M (2019) Biochemical and molecular characterization of cowpea landraces using seed storage proteins and SRAP marker patterns. *Saudi J Biol Sci* 26:74–82
- Arefrad M, Babaian Jelodar N, Nematzadeh G, Karimi M, Kazemitabar S (2014) Assessment of seed storage protein composition of six Iranian adopted soybean cultivars [*Glycine max* (L.) Merrill.]. *J Plant Mol Breed* 2(1):29–44
- Badr A, El-Sherif N, Aly S, Ibrahim SD, Ibrahim M (2020) Genetic diversity among selected *Medicago sativa* cultivars using inter-retrotransposon-amplified polymorphism, chloroplast DNA barcodes and morpho-agronomic trait analyses. *Plants* 9:995
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254

- Corts MM, Martinez MC (2000) Variation of PGM and IDH isozymes for identification of alfalfa varieties. *Euphytica* 112:137–143
- Darvishi-Zaidabadi D, Mirzaie-Nodoushan H, Hesamzadeh H, Madad-Arefi H (2000) Seed storage electrophoresis for investigating existing variation between alfalfa (*Medicago sativa*) Cultivars. *Iran J Rangel For Plant Breed Genet Res* 5:111–124. <https://doi.org/10.22092/ijrfpbgr.2000.115891>
- Fareghi SH, Farshadfar M, Farshadfar E (2007) Study of chemical composition and nutrition value of perennial Lucerne (*Medicago sativa* L) and genetic diversity based on SDS-PAGE markers. *Iran J Rangel For Plant Breed Genet Res* 15:196–210
- Fyad-Lameche F, Bellatar G, Bouabdallah S, Yahia N (1996) Between and within species variation in annual *Medicago* species Cahiers Options Mediterraneeennes (CIHEAM)
- Golan SJ (2013) The study of saponin(s) as resistance factors against alfalfas weevil (*Hypera postica* Gyll.) larva feeding with the assessment of digestive enzyme activity using TLC. Bu-Ali Sina University, Hamadan
- Haidari P, Mehrabi AA, Nasrollah NGAA (2014) Genetic diversity of Balm (*Melissa officinalis* L.) landraces and genetic relationship within and between them using ITS markers
- Hamouda M (2019) Molecular analysis of genetic diversity in population of *Silybum marianum* (L.) Gaertn. *Egypt J Genet Eng Biotechnol* 17:12
- Hou W, Zhang X, Shi J, Liu Y (2015) Genetic diversity analysis of faba bean (*Vicia faba* L.) germplasm using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Genet Mol Res* 14:13945–13953
- Ismaili A, Mojiri F, Hosseini SZ (2013) Use of intron-exonic marker in assessment of genetic diversity of two subspecies of *Thymus daenensis*. *Taxon Biosyst* 5:41–54
- Javaid A, Ghafoor A, Anwar R (2004) Seed storage protein electrophoresis in groundnut for evaluating genetic diversity. *Pak J Bot* 36:25–30
- Kakaei M, Kahrizi D (2011) Study of seed proteins pattern of brassica napus varieties via sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Int Res J Biotechnol* 2:026–028
- Kakaei M, Mazahery-Laghab H (2014) Evaluation of alfalfa (*Medicago sativa* L.) germplasm using multivariate statistical analysis. *Iran J Rangel For Plant Breed Genet Res* 22:125–132
- Kidwell KK, Austin DF, Osborn TC (1994) RFLP evaluation of nine *Medicago* accessions representing the original germplasm sources for North American alfalfa cultivars. *Crop Sci* 34(1):230–236
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Lawrence G, Moss H, Shepherd K, Wrigley C (1987) Dough quality of biotypes of eleven Australian wheat cultivars that differ in high-molecular-weight glutenin subunit composition. *J Cereal Sci* 6:99–101
- Musavi Z, Tahernezhad Z, Zamani M, Imamjome A (2010) Studying of glutenin subunits diversity in *aegilops tauschii* accessions by SDS-Page method
- Nisar M, Ghafoor A, Khan MR, Ahmad H, Qureshi AS, Ali H (2007) Genetic diversity and geographic relationship among local and exotic chickpea germplasm. *Pak J Bot* 39:1575–1581
- Shamustakimova A, Klimenko I (2019) Genetic diversity of alfalfa cultivars of Russian breeding revealed by microsatellite markers
- Stoyanova S, Boller B (2010) Seed protein electrophoresis for assessment of genetic variation within genotypes of meadow fescue (*Festuca pratensis* Huds). *Czech J Genet Plant Breed* 46:S75–S81
- Valizadeh M (2001) Seed storage protein profile of grain legumes grown in Iran, using SDS-PAGE. *J Agric Sci Technol* 3(4):287–292
- Wang X, Yang X, Chen L, Feng G, Zhang J, Jin L (2011) Genetic diversity among alfalfa (*Medicago sativa* L.) cultivars in Northwest China. *Acta Agric Scand Sect B Soil Plant Sci* 61:60–66
- Xi J, Wang X, Li S, Zhou X, Yue L, Fan J, Hao D (2006) Polyethylene glycol fractionation improved detection of low-abundant proteins by two-dimensional electrophoresis analysis of plant proteome. *Phytochemistry* 67(21):2341–2348
- Zivkovic B, Radovic J, Sokolovic D, Siler B, Banjanac T, Strbanovic R (2012) Assessment of genetic diversity among alfalfa (*Medicago sativa* L.) genotypes by morphometry, seed storage proteins and RAPD analysis. *Ind Crops Prod* 40:285–291