

# Using Morphological and Microsatellite (SSR) Markers to Assess the Genetic Diversity in Alfalfa (*Medicago sativa* L.)

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**Abstract**—Utilization of diverse germplasm is needed to enhance the genetic diversity of cultivars. The objective of this study was to evaluate the genetic relationships of 98 alfalfa germplasm accessions using morphological traits and SSR markers. From the 98 tested populations, 81 were locals originating in Europe, 17 were introduced from USA, Australia, New Zealand and Canada. Three primers generated 67 polymorphic bands. The average polymorphic information content (PIC) was very high (> 0.90) over all three used primer combinations. Cluster analysis using Unweighted Pair Group Method with Arithmetic Means (UPGMA) and Jaccard's coefficient grouped the accessions into 2 major clusters with 4 sub-clusters with no correlation between genetic and morphological diversity. The SSR analysis clearly indicated that even with three polymorphic primers, reliable estimation of genetic diversity could be obtained.

**Keywords**—genetic diversity, *Medicago sativa* L., morphological traits, SSR markers

## I. INTRODUCTION

THE genus *Medicago* is distributed worldwide and consists of approximately 83 species [1]. The cultivated alfalfa (*Medicago sativa* L.) is an autotetraploid, ( $2n = 4x = 32$ ), cross-pollinated (allogamous) and seed – propagated species [2]. These factors contribute to the genetic complexity of alfalfa. It is the most cultivated forage legume, with about 32 million ha over the world. Its agronomical interest is based on its high protein content, suitable feeding value and favorable environmental impact (perenniality and no nitrogen fertilizer required). Morphological characteristics are the strongest determinants of the agronomic value and taxonomic classification of plants. For legumes, growth habit, flower color, leaf shape, pod and seed shape are usually used as indexes to estimate diversity between species. Compared with others means, morphological evaluations are direct, inexpensive and easy. However, errors can arise, furthermore, morphological estimations are more dependent on environment and are more subjective than other measurements.

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On the other hand, DNA-based molecular markers allow a more precise and environment-independent way of studying genetic diversity. Several studies have been done on alfalfa using DNA-based markers like AFLP [3], RAPD [4], [5], ISSR [6], RFLP and SSR marker [4], [7], [8], [9].

Among these markers, SSR is accepted as an effective means of assessing the level of variation among alfalfa populations and describing the relationships among various alfalfa genotypes.

Simple sequence repeats (SSR), also known as microsatellite DNA markers, is polymerase chain reaction (PCR)-based genetic markers. Compared with other markers, SSR has many advantages: highly polymorphic, co-dominant inherited, reproducible and reliable, therefore they have been proposed as one of the most suitable markers for the assessment genetic diversity of plants and animals.

In the present study, we examined 98 population of genus *Medicago* with 3 SSR markers and 50 morphological traits. The objectives of the current study were to estimate the genetic diversity among alfalfa populations, and to compare morphological and genetic diversity among these populations.

## II. MATERIALS AND METHODS

### A. Plant Material and Field Conditions

Varieties used for microsatellite polymorphism analysis came from 24 states of the world. Genetic variability was detected in 91 origins of *Medicago sativa*, 6 origins of *Medicago varia* and 1 origin of *Medicago falcata*. (Table I). The test material was obtained from genotypes collection of Research Institute for Fodder Crops, Ltd., stored in a central Gene Bank in the Crop Research Institute Prague-Ruzyně (Czech Republic). The plant material was grown in the field Research Institute for Fodder Crops, Ltd. in Troubsko. The evaluations were made both in individual plantings and in the stand. In individual plantings 10 plants of each accession were evaluated. Stands were evaluated in the parcels of 10m<sup>2</sup>, established by the method of randomised blocks.

### B. DNA Extraction

Materials for DNA isolation were obtained from a set of 20 to 25 plants of each populations at the 3 to 4 leaf stage. The samples were ground into a very fine powder using liquid nitrogen. Total genomic DNA was extracted using the commercial GenElute™ Plant Genomic DNA Miniprep Kit (Sigma, Germany).

TABLE I  
LIST OF 98 MEDICAGO CULTIVARS USED FOR GENETIC DIVERSITY STUDIES AND THEIR ORIGIN

| Entry No. | Accession Name   | Origin | Species | EntryNo. | Accession Name   | Origin | Species |
|-----------|------------------|--------|---------|----------|------------------|--------|---------|
| 1         | Mnogolistna      | BGR    | sativa  | 50       | Vogherese        | ITA    | sativa  |
| 2         | Victoria         | BGR    | sativa  | 51       | KM Norbert       | HUN    | sativa  |
| 3         | Naraganset       | USA    | varia   | 52       | Euver            | FRA    | sativa  |
| 4         | Kákai legelo     | HUN    | sativa  | 53       | Vertus           | SWE    | sativa  |
| 5         | Centauro         | ITA    | sativa  | 54       | Cimarron         | AUS    | sativa  |
| 6         | Midi             | NLD    | sativa  | 55       | Szapko           | HUN    | sativa  |
| 7         | NS Banat ZMS II  | YUG    | sativa  | 56       | Saranac AR       | USA    | sativa  |
| 8         | Medina           | FRA    | sativa  | 57       | Proteina 1       | BGR    | sativa  |
| 9         | SVK 21           | SVK    | sativa  | 58       | Krajina          | YUG    | sativa  |
| 10        | SVK 39           | SVK    | sativa  | 59       | Baron            | USA    | sativa  |
| 11        | Préri            | HRV    | sativa  | 60       | Prescot          | DNK    | sativa  |
| 12        | T. pastbišćenaja | SUN    | varia   | 61       | Nugget           | USA    | sativa  |
| 13        | Paula            | DDR    | sativa  | 62       | Sverre           | SWE    | sativa  |
| 14        | Natsuwakaba      | JAP    | sativa  | 63       | Resistador       | USA    | sativa  |
| 15        | Gigante Romea    | ITA    | sativa  | 64       | Maris Phoenix    | GBR    | sativa  |
| 16        | Tula             | POL    | varia   | 65       | Grassland Oranga | NZL    | sativa  |
| 17        | Julus            | SWE    | sativa  | 66       | Lesina           | SWE    | sativa  |
| 18        | Isis             | DNK    | sativa  | 67       | Nadezda 2        | BGR    | sativa  |
| 19        | Vali             | SVK    | sativa  | 68       | Vela             | DNK    | sativa  |
| 20        | Gabika           | SVK    | sativa  | 69       | Orca             | FRA    | sativa  |
| 21        | Weibul B7        | SWE    | sativa  | 70       | Europe           | FRA    | sativa  |
| 22        | Tango            | FRA    | sativa  | 71       | Palava           | CZE    | sativa  |
| 23        | KPTS 2           | HUN    | sativa  | 72       | Zuzana           | CZE    | sativa  |
| 24        | Barlydia         | NLD    | sativa  | 73       | Preserve         | USA    | sativa  |
| 25        | Jitka            | CZE    | sativa  | 74       | Morava           | CZE    | sativa  |
| 26        | Vlasta           | CZE    | sativa  | 75       | Belfeuil         | FRA    | sativa  |
| 27        | Niva             | CZE    | sativa  | 76       | Verko            | DEU    | sativa  |
| 28        | Bardesi          | NLD    | sativa  | 77       | Hodonínka        | CZE    | sativa  |
| 29        | Radius           | POL    | varia   | 78       | Anik             | CAN    | falcata |
| 30        | Casalina         | ITA    | sativa  | 79       | Equipe           | ITA    | sativa  |
| 31        | Express          | USA    | sativa  | 80       | Leonicena        | SUN    | sativa  |
| 32        | Panonia          | HRV    | sativa  | 81       | Sirosal1         | AUS    | sativa  |
| 33        | Magnat           | ROM    | sativa  | 82       | Eugenia          | ITA    | sativa  |
| 34        | Malvina          | LTU    | sativa  | 83       | Team             | USA    | sativa  |
| 35        | Os-11            | CRO    | sativa  | 84       | Hybride de Crécy | FRA    | sativa  |
| 36        | Jarka            | CZE    | sativa  | 85       | Du Puits         | FRA    | sativa  |
| 37        | Jersey           | FRA    | sativa  | 86       | Slavonka         | YUG    | sativa  |
| 38        | Bistra           | SLO    | sativa  | 87       | C 3              | USA    | sativa  |
| 39        | Daphne           | FRA    | sativa  | 88       | Agate            | USA    | sativa  |
| 40        | Precedent        | USA    | sativa  | 89       | Lutece           | FRA    | sativa  |
| 41        | POC-4/82         | POL    | varia   | 90       | Luxin            | ROM    | sativa  |
| 42        | Mercedes         | FRA    | sativa  | 91       | Zarnica          | SUN    | sativa  |
| 43        | Planet           | DEU    | sativa  | 92       | Sabilt           | GBR    | sativa  |
| 44        | Biruté           | LTA    | sativa  | 93       | Gamma            | FRA    | sativa  |
| 45        | Pella            | GRE    | sativa  | 94       | Ondava           | SVK    | sativa  |
| 46        | Meldore          | FRA    | sativa  | 95       | Multileaf        | USA    | sativa  |
| 47        | Kometa           | POL    | varia   | 96       | Advantage        | USA    | sativa  |
| 48        | Altiva           | ESP    | sativa  | 97       | Resis            | DNK    | sativa  |
| 49        | Adorna           | NLD    | sativa  | 98       | Přerovská        | CZE    | sativa  |

### C. SSR Assays

Three SSR primer pairs were selected from the study of [8], [11] and [12]. PCR was performed for a 20  $\mu$ l total volume containing 1x reaction buffer, 100  $\mu$ M dNTPs, 10  $\mu$ M of each SSR primer, 1.5 mM MgCl<sub>2</sub>, 2  $\mu$ l genomic DNA and 0.9 units of *Taq* polymerase.

Reaction were performed in a Techne gradient thermocycler programmed for an initial denaturation at 95°C for 4 min. followed by 30 cycles at 95°C for 30 s, annealing temperature at 55°C for 30 s and extension at 72°C for 30 s, after which followed another 10 cycles with annealing temperature at 53°C for 30 s and then a final extension step at 72°C for 10 min. The forward primers of each pair were labeled with a fluorescent dye (6 – FAM). The fragment analysis was performed on an ABI Prism@3130 Genetic Analyser (Applied Biosystems). The sizes of the alleles in base pairs were estimated using GeneMapper analysis software (version 4.0; Applied Biosystems).

### D. Statistical Analysis

All the genotypes were scored for the presence and absence of the SSR bands. And the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. To compare two different techniques, such genetic parameters were computed as the number of polymorphic bands, and average number of alleles per locus. To calculate the expected heterozygosity ( $H_e$ ) and the polymorphism information content (PIC) was used PIC Calculator Extra free software [13] (<http://www.genomics.liv.ac.uk/animal/pic.html>).

The similarity matrix was created from binary data using Jaccard's [14] coefficient for SSR. Based on similarity matrix cluster analysis, Unweighted Pair Group Method with Arithmetic Means (UPGMA) was performed using FreeTree software v. 0.9.1.50 [15] (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

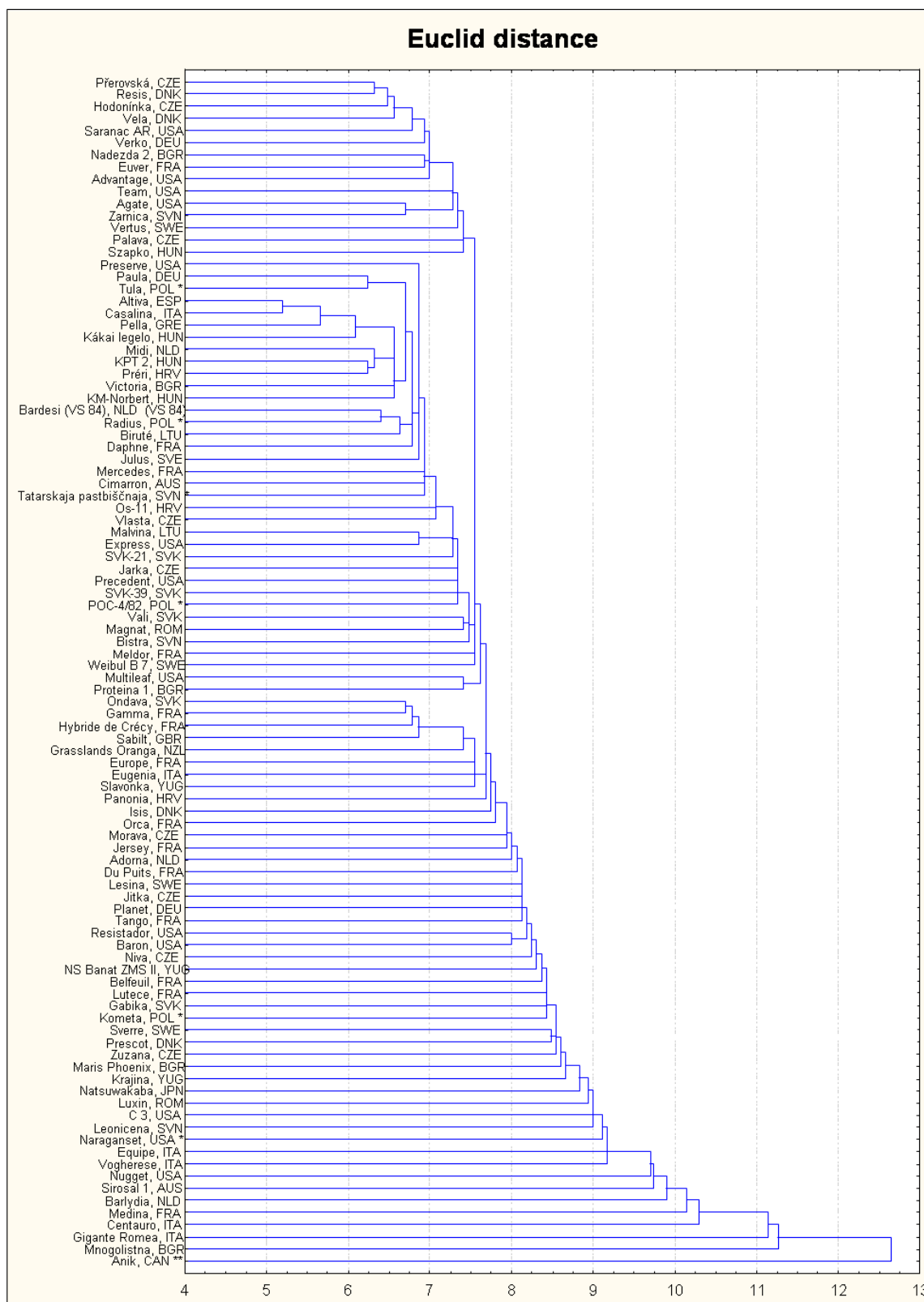
Cluster analysis was computed by help of the programme Statistica 7.1 [16] (StatSoft, Inc. 1984 – 2005). For calculation the Euclidean distances, Unweighted Pair Group Method with Arithmetic Means (UPGMA) was used.

The matrices of dissimilarity coefficients from the cluster analysis based on morphological and that of molecular data were compared by Mantel test. The test was performed in the software XLSTAT version 2012.3.01 [17] (Addinsoft, Inc. 1995 – 2012).

## III. RESULTS AND DISCUSSION

### A. Morphological Analyses

Dendrogram of morphological characteristics used for the evaluation of the 98 *Medicago* accessions was constructed. Fig. 1 represents the dendrogram based on morphological data, it shows separation of items into 2 main clusters with population Anik (CAN) comprising a singular group and 2 sub-clusters. The first sub-cluster comprised accessions from Proteina 1 (BGR) to Přerovská (CZE) and the second sub-cluster involved items from Ondava (SVK) to Mnogolistna (BGR). The genetic diversity based on morphological characters ranged from 6.08 to 20.64. The highest genetic similarity (6.08) was found between Altiva (ESP) and Casalina (ITA) accession and the highest genetic dissimilarity (20.64) was found between Anik (CAN) and Niva (CZE) items. Anik (CAN) is a species of *M. falcata*, with the lowest 1000-seed weights and total hay yield of stand. The highest green biomass weight per plant and seed weight per plant has been found in the Czech varieties Niva, Jarka, Vlasta and Jitka. In addition, the highest level of resistance to *alfalfa mosaic virus* (AMV) was found in Niva, Jarka and Vlasta cultivars. The highest total hay yield of stand was observed in variety Zuzana (CZE) and the highest green biomass weight was found in Lesina (SWE) cultivar.



\* *M. varia*  
 \*\* *M. falcata*

Fig. 1 Dendrogram generated using UPGMA, showing relationships between 98 *Medicago* genotypes, using morphological data

*B. SSR analyses*

In this study, three simple sequence repeat (SSR) primer sets (Table II) were used to detect and estimate genetic polymorphism based on co-dominant marker system. Used microsatellites displayed a high level of polymorphism in the present study. A total of 67 polymorphic bands were amplified using these SSR markers.

The number of alleles per locus ranged from 19 for B21E13 to 24 for Afctt1 and MTIC297, with an average 22.3 alleles. The average number of alleles in this study (22.3) is comparable to those from previous studies of genetic diversity between *Medicago* germplasm collection, for example [18] had reported 16.7. In contrast to work by [19] which had found 8.5 alleles per locus on average, with the number of alleles per SSR locus ranging from 4 to 14. The PIC and H<sub>e</sub> value for all three used SSR markers was very high (> 0.90) (Table II).

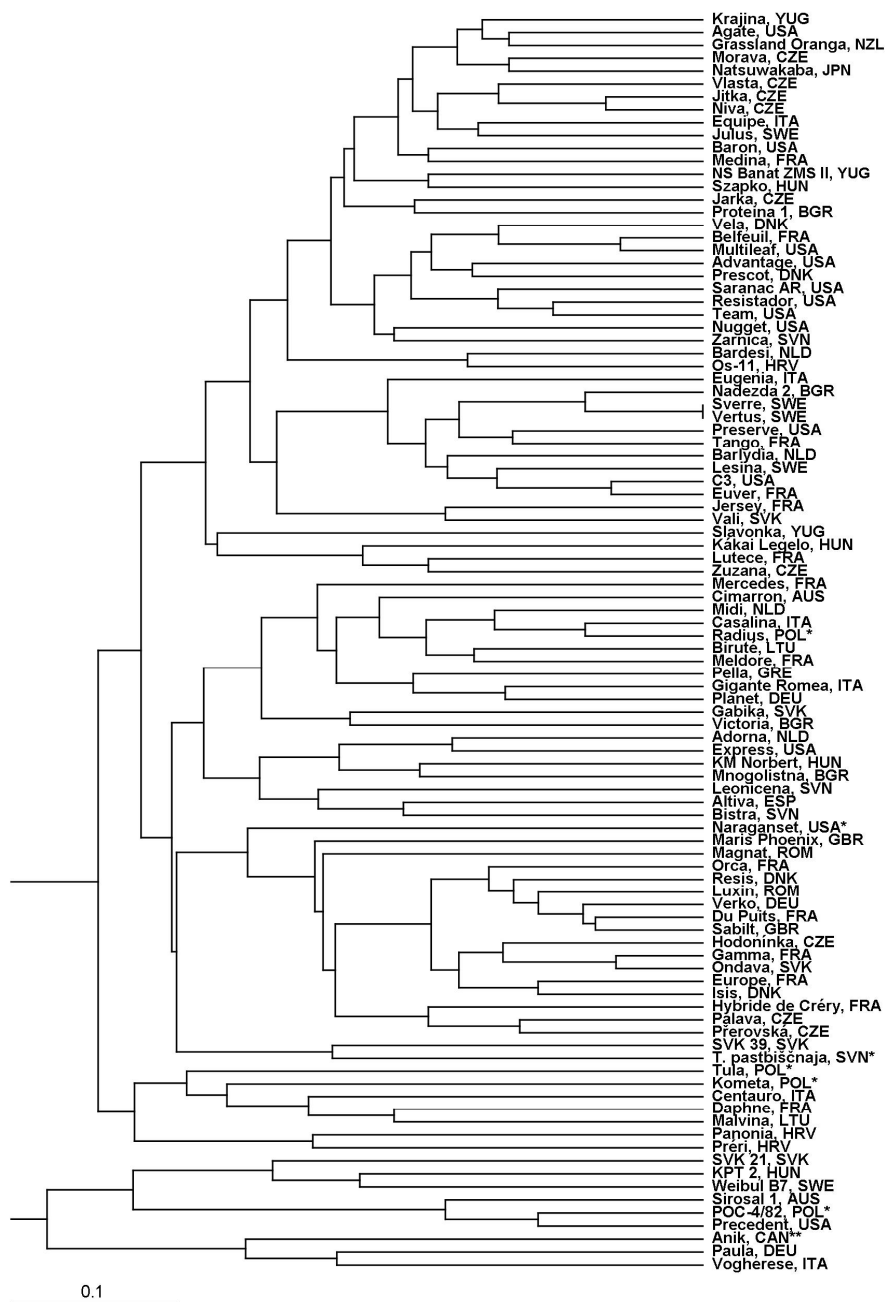
TABLE II  
 NUCLEOTIDE SEQUENCES OF 3 POLYMORPHIC PRIMERS AND PIC AND H<sub>e</sub> VALUES IN SSR ANALYSIS

| Locus       | Core motif                            | Forward primer        | Reverse primer      | PIC           | H             |
|-------------|---------------------------------------|-----------------------|---------------------|---------------|---------------|
| AFctt1 (D)  | (CTT) <sub>9</sub> (CAA) <sub>3</sub> | cccatcatcaaacatttca   | ttgtgattggaacgagt   | <b>0.9121</b> | <b>0.9178</b> |
| MTIC297 (T) | (TAC) <sub>5</sub>                    | ctaagctttggccatgtatc  | tgaatgagtttgactgagg | <b>0.901</b>  | <b>0.9082</b> |
| B21E13 (J)  | (GA) <sub>13</sub>                    | gccgatggtaactaatgtagg | aaatcttgcttctctcag  | <b>0.9163</b> | <b>0.9214</b> |

D – [8], T – [11], J – [12]

Basing on SSR markers an UPGMA analysis was performed. The resulting dendrogram (Fig. 2) defines the genomic relationships among analyzed cultivars. The Jaccard's coefficient varies from 0.029 (high genomic dissimilarity) to 1 (full genomic similarity), thus demonstrating the high polymorphism of the analyzed genotypes. The results showed that lowest genetic distance was recorded between Sverre (SWE) and Vertus (SWE) populations and highest genetic distance was calculated between Express (USA) and Vogherese (ITA) population.

The dendrogram based on molecular data representing the relationship between individuals (Fig. 2) did not divide all genotypes into distinct groups resembling the analysed alfalfa varieties. The accessions clustered into 2 major clusters with 4 sub-clusters. The first sub-cluster comprised accessions from Vogherese (ITA) to Anik (CAN). The second cluster involved items from Precedent (USA) to SVK 21 (SVK). The third sub-cluster included accessions from Préri (HRV) to Tula (POL). The fourth sub-cluster is divided into additional two sub-cluster, which involved accessions from included varieties from T. pastbiščajna (SVN) to Mercedes (FRA) and varieties from Zuzana (CZE) to Krajina (YUG). This group included Sverre (SWE) and Vertus (SWE) accessions with 100% similarity.



\* *M. varia*  
 \*\* *M. falcata*

Fig. 2 UPGMA dendrogram of 98 *Medicago* genotypes based on genetic similarity obtained using 3 SSR markers

In this study, we investigated the morphological and genetic diversity of *Medicago* populations. The main aim of our study was to evaluate the genetic diversity of *Medicago* genotypes and to compare morphological distance with genetic distance.

We performed correlation analysis between the morphological and genetic diversity of 98 *Medicago* populations. The matrices were compared using Pearson's correlation coefficient ( $r = 0.094$ ), which was very low. In our study, no significant correlation was detected between observed patterns of morphological and molecular variation. A low correlation between phenotypic distance and distance measured using SSR markers had been obtained in the research of [20] and [18].

The same results were documented in many other crops ([21] – *Trifolium pratense*, [22] – *T. repens*, [23]– *Vitis vinifera*). In agreement with previous studies, phenotypic distance and genetic distance did not define the same pattern of population clustering. This may have resulted from the random selection of DNA markers and the environmental dependence of morphological traits, as well as uncertainty regarding whether the alleles correlated with the phenotypic characteristics studies. The SSR markers used in this study were highly polymorphic and efficient in revealing the level of genetic diversity present in the populations studied.

Our results indicate that markers with large number of alleles are informative for population studies. The high average number of alleles per locus per plant in alfalfa could be due to very high level of heterozygosity and allogamous nature of cultivated alfalfa.

In conclusion, the study confirmed that genetic and morphological diversity work in different way to determine the relationships among populations.

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