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Genetic diversity of alfalfa (*Medicago* spp.) estimated by molecular markers and morphological characters

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Abstract

Background and Purpose: *Genetic diversity of initial selection materials is essential for successful breeding and creation of new cultivars. The objectives of this research were to study genetic diversity among alfalfa (Medicago spp.) cultivars/population with a different geographical origin by RAPD markers and morphological characters, to determine genetic variation among and within materials using RAPD markers, and to compare results based on molecular markers and morphological characters.*

Materials and Methods: *Fourteen European, Australian, South and North American* M. sativa *and* M. media *cultivars and one French wild population* M. falcate *were studied. Analyses were conducted on 20 randomly chosen individual plants of each cultivars/population. Genetic distances were calculated on the basis of RAPD data and morphological characters. Cluster analysis and AMOVA were shown for molecular data. Statistical analyses were performed in SAS 8.0, Arlequin 2.0 and NTSYS-pc 2.20 software.*

Results and Conclusions: *The six RAPD primers generated 90 polymorphic bands across the 300 individual plants analyzed. Genetic distance among cultivars/population ranged from 0.28 to 0.40. Cluster analysis clearly separated Maron (*M. falcata*) population from all* M. sativa *and* M. media *cultivars which were grouped into two main clusters. Most of the genetic variability estimated by AMOVA was attributed to variation among individuals within cultivars (91.86%). Eight morphological characters, used for morphological analysis, were not sufficient to determine the differences among cultivars. Significant correlations between morphological and molecular distances were not observed. The obtained data suggest that RAPD markers could be useful and powerful tools for estimation of genetic diversity among diverse alfalfa germplasm.*

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a widely grown legume and one of the most important forage species throughout the world. It yields forage of high nutritional quality and has positive influences on the environment. It is a perennial species that in usually grown at cultivated land for four to five years and under no-stress agricultural conditions gives high yield of green mass which may be used in a number of different forms (green mass, hay, silage, greenchop, briquette, pellets, etc.). Alfalfa is autotetraploid (2n = 4x = 32), allogamous and a seed-propagated species. These factors contribute to genetic complexity of alfalfa

at both individual and population levels *(1)*. The most frequent breeding methods applied to alfalfa involve different forms of mass selection, phenotypic recurrent selection and development of synthetic populations. Information about germplasm diversity and relationships among elite breeding materials is of fundamental importance in plant breeding *(2)*. This is epecially true for species like alfalfa which suffers severe inbreeding depression. Genetic diversity of initial selection materials is essential for successful breeding and creation of new cultivars. For the estimates of genetic diversity, different criteria, as morphological, agronomic and physiological characters, pedigree records, molecular markers or a combination of criteria are used.

Molecular markers, unlike from traditional phenotypic markers, detect variability and differences among and within cultivars directly at the DNA level. As they are independent of environmental factors, they enable a more precise determination of genetic relatedness. Different molecular marker types have been used to assess genetic diversity in alfalfa: restriction fragment length polymorphism (RFLP) *(3, 4)*, random amplified polymorphic DNA (RAPD) *(5–7)*, simple sequence repeat (SSR) *(8)*, sequence related amplified polymorphisms (SRAP) *(9)*, and amplified fragment length polymorphism (AFLP) *(10)*. Since no previous knowledge about genome is required, RAPD genetic markers allow a rapid analysis of the polymorphism in many individuals. They have been used widely in analyses of genetic structure of alfalfa and of numerous other forage crops such as red clover *(11, 12)*, white clover *(13)*, perennial ryegrass *(14, 15)*, and *Dactylis glomerata (16)*.

Erosion of diversity in most of the cultivated species emphasizes the need to collect and investigate new germplasm as genetic resources for future breeding programs. Alfalfa is distributed worldwide and grown in highly contrasting environments. This extensive geographical adaptation promotes genetic variation and gives us the opportunity to use diverse gene pools. The aims of this research were to study genetic diversity among alfalfa cultivars/population with different geographical origin by RAPD markers and morphological characters, to determine genetic variation among and within cultivars using RAPD markers, and to compare results based on molecular markers and morphological characters.

MATERIAL AND METHODS

Plant material, experimental set up and DNA preparation

Fourteen cultivars and one population of *Medicago* spp. with different geographical origin were studied. *Medicago sativa* L. cultivars included European, Australian, South and North American cultivars. European cultivars included two Croatian cultivars (Vuka and OS-93 created at Osijek Agricultural Institute), two French cultivars (Magali and Europe, obtained from the INRA Unite de Genetique et Ammeliration des Plantes Fourrageres de Lusignan), one Italian cultivar, Classe, obtained from Dr. Fabio Veronesi (Istituto di Miglioramento Genetico Vegetale, Perugia), and two Swedish cultivars (Sverre and Pondus, obtained from the Nordic Gene Bank and Svalof Weibull). Two Australian cultivars, Genesis and Prime, were obtained from the South Australian *Medicago* Genetic Resource Centre, SARDI, Waite Campus, Urrbrae, Australia. South American cultivars Barbara and Victoria were obtained from Dr. Daniel Basigalup (INTA Estacion Experimental Agropecuaria Manfredi, Argentina). Two North American cultivars Blazer XL and Magnum V-Wet were provided by Dr. Dan Undersander (University of Wisconsin, Madison, USA). *Medicago falcata* L. species included one French wild population, Maron provided by Dr. Julier Bernadette (INRA, Lusignan). Polish cultivar, Radius (*Medicago media* L.), was obtained from the IHAR National Centre for Plant Genetic Resources at Radzikow. A seed of the 15 alfalfa cultivars/population for subsequent field trial was sown in peat pellets (Jiffy pots no. 7, Jiffy Products Ltd, Norway) and maintained in the glasshouse up to the development of four to five leaves per each plant (mid April 2004). Plants were transplanted to the field at location Osijek (lat 45°32'N, long 18°44'E, altitude 90 m). Experimental design was a randomized complete block with three replications. Each experimental plot (1×5.5 m) included 36 spaced plants (0.50×0.50 m) of each cultivar/population. For RAPD analyses, 20 randomly chosen individual plants of each cultivar/population were selected. Young, healthy and clean trifoliate leaves were harvested from each plant and preserved at – 80 °C up to lyophilization. Genomic DNA of 300 individuals in total was isolated by ABI PRISM^{TM} 6100 Nucleic Acid PrepStation (Applied Biosystems, Fostert City, California, USA) complying with the Protocol for isolation of DNA from plant tissue – Quick reference card. The presence of genomic DNA with high molecular weight was verified using 1% agarose gel in 1x TBE buffer. DNA was diluted to the concentration of 2 ng DNA/μl and used for RAPD-PCR amplification.

RAPD analyses

PCR reactions were performed in total volumes of 25 μl under final conditions of 1x PCR reaction buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of *Taq* DNA polymerase (Applied Biosystems), 0.3 μM primer (Invitrogen, Carlsbad, California, USA) and 20 ng of genomic DNA. Reactions were performed in GeneAmp 9700 thermocycler (Applied Biosystems). The cycling regime was 92 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min over 42 cycles. A final extension step at 72 °C for 6 min was performed after 42 cycles. In order to test amplification profiles for polymorphism, readability and reproducibility, an initial screening of 36 RAPD decamer primers was performed using five individual plants of the cultivars Prime, Magnum V-Wet and Maron population. Six primers were selected for analysis after screening (Table 1). DNA products were separated by electrophoresis on 1.5% agarose gels that contained SYBR Safe (Invitrogen) DNA gel

TABLE 1

Nucleotide sequence of the primers used in RAPD analysis, total number of bands per primer and the number of polymorphic bands per primer.

Primer	Sequence (5'→3')	Total number of RAPD bands	Number of polymorphic bands
RAPD 1	CGTCTGCCCG	16	14
RAPD 4	CTGGCGGCTG	22	19
RAPD 8	GTGCGTCCTC	16	15
RAPD 11	CAAACGGCAC	15	14
RAPD 35	GGGCATCGGC	18	18
D-36	GTCCACACGG	11	10
Total		98	90

stain for visualization. The gels were soaked in 1x TBE buffer at 120V for 3 h. Products were visualized by UV transilluminator and images were taken with a Kodak DC290 digital camera. Gel images were analyzed with Kodak 1D Image Analysis Software (version 3.6, Scientific Imaging Systems, Eastman Kodak Company, Rochester, N.Y.). 100 base pair (bp) DNA ladder (Amersham Biosciences, Piscataway, NJ, USA) was used as a standard.

Morphological characters

Morphological characters were evaluated on 20 individual plants of each cultivar/population previously selected for RAPD analyses. Plants were described at the field combining Guidelines for the conduct of tests for distinctness, homogeneity and stability of lucerne *(17)* and Forage Legume Descriptors *(18)*. The following characters were evaluated: 1) growth habit was scored at the beginning of flowering on the scale from 1 = erect to 9 = prostrate, 2) plant color was determined three weeks after the first spring cut from 3 = light green to 7 = dark green, 4) height of the longest stem was measured from the first stem internodes to the top of inflorescence (cm), 5) regeneration was assessed within two weeks after the previous cut by individual measurements of the plant height (cm). The third leaf below the first inflorescence in early flower bud stage was scored for 6) shape from 3 = elongated to 7 = round, 7) length of central leaflet from 1 = very short to 9 = very long, and 8) width of central leaflet from 1 = very narrow to 9 = very broad.

Statistical analysis

RAPD bands within the range of 200-2300 base pairs were scored for presence (1) or absence (0) and entered into a data matrix. Monomorphic bands were excluded from data analysis. To reduce estimation bias, amplified bands were tested according to Lynch and Milligan *(19)*. The RAPD binary matrix for 20 individuals of each cultivar/population was used to calculate modified Rogers distance (MRD) *(20)* as:

$$d_{ij}^{RAPD} = \sqrt{\frac{\sum (Y_{ia} - Y_{ja})^2}{A}}$$

where *Yia* and *Yja* denote the frequency of a band *a* in the cultivars *i* and *j*, respectively, and summation is over the bands (a = 1, 2,.....A, with A = 90). In this case, MRD is proportional to the Euclidean distance based on band frequencies. MRD was calculated using SAS 8.0 software *(21)*. The distance matrix was used as input data for cluster analysis based on the unweighted pair-group method of arithmetic averages (UPGMA) with NTSYS-pc program ver. 2.20 *(22)*. The MRD matrix was used to produce an analysis of molecular variance (AMOVA) in order to estimate components of variance attributable to differences among and within cultivars *(23)*. AMOVA was performed using the Arlequin program ver. 2.0 *(24)*.

Data for morphological variables were standardized as $Y_m = (x_m - {}_m)/ sd_{xm}$ where x_m is the m-th morphological character before standardization (m = 1....M, with M = 8), y_m standardized character, ${}_m$ the mean of x_m and sd_{xm} standard deviation of x_m. The morphological distance between cultivar i and j were calculated as:

$$d_{ij}^{Morph} = \frac{\left[\sum_{m=1}^{M} (Y_{im} - Y_{jm})^2\right]^{1/2}}{4M}$$

where M is the number of morphological characters, and 4M the normalizing constant *(20)*. The UPGMA was made on the basis of a distance matrix.

The correspondence between pairs of matrices based on the morphological and molecular distances was tested with Mantel Z-statistic *(25)*. The procedure examines the matrix correspondence by taking matrices together and plotting one against the other, element by element. This test gives the product moment correlation r, and statistic Z in order to measure the degree of relation between two matrices. Significance of Z was assessed by comparing the observed Z value with a critical Z value obtained by calculating Z for 1 matrix with 1000 permuted variants of the second matrix. All calculations were performed by NTSYS-pc program ver. 2.20 *(22)*.

RESULTS

Out of 36 primers tested, 30 primers (83.33%) gave amplification products. Six of them (20.00%) generated stable and reproducible bands. With these six primers, a total of 90 polymorphic bands for 300 individuals of 15 alfalfa cultivars/population were generated. The average number of polymorphic bands per primer was 15. The highest number of polymorphic bands (21.11% of total number of polymorphic bands) was achieved with primer RAPD 4 (Table 1).

As an example of amplification, PCR products of 20 individuals of Magnum V-Wet cultivar obtained with primer RAPD 35 are shown by Figure 1. The total num-

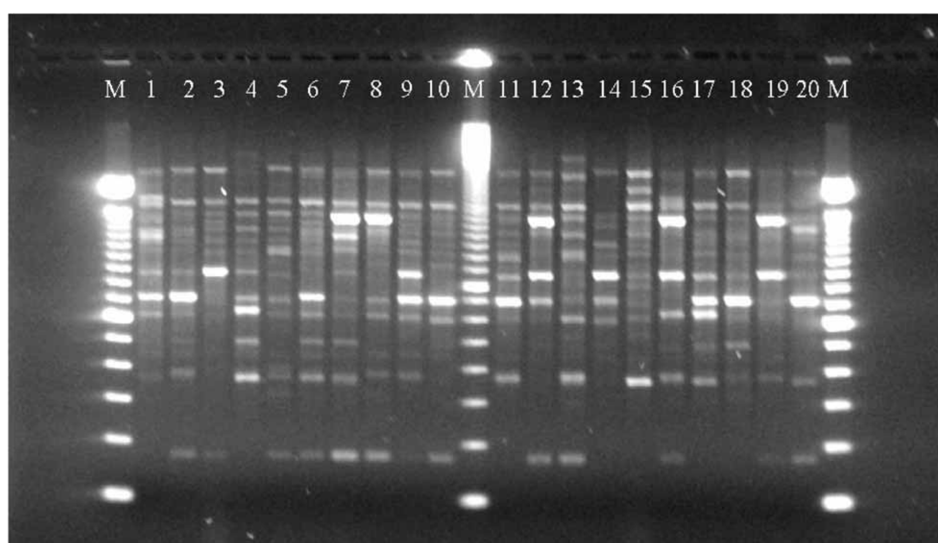

Figure 1. *RAPD patterns for 20 individuals of cultivar Magnum V-Wet generated by primer RAPD 35 (numbers indicate individual plants, M–molecular size (100-pair base DNA ladder, Amersham Biosciences).*

ber of polymorphic bands identified within each cultivar/population ranged from 52 in Maron population (83.87% of total band number) to 74 in Magnum V-Wet cultivar (90.24% of total band number), with an average of 63 bands (87.41% of total band number). Results are shown in Table 2. Specific bands were identified for Maron population (two bands: 1180 bp generated by primer RAPD 1 and 570 bp generated by primer RAPD 8) and Radius cultivar (one band: 1770 bp generated by primer RAPD 4). The band with size 850 bp generated by RAPD 4 was identified only in samples of Maron population (in 45% of all plants analyzed) and Radius cultivar (in 40% of all plants analyzed).

The French population Maron (*M. falcata*) was genetically distant from the other materials studied. Compared with *Medicago sativa* cultivars, an average genetic distance was 0.38. The smallest genetic distance was observed between Argentinian cultivar Victoria and French cultivar Europe (0.28), and the largest between population Maron and Argentinian cultivar Barbara (0.40) (Table 3). Genetic distance values were used to construct a dendrogram (Figure 2).

The French population Maron (*M. falcata*) was the most distant and clustered separately from the remaining *M. sativa* and *M. media* alfalfa cultivars, which were grouped into two main clusters. The first cluster included Australian cultivars Genesis and Prime, Swedish cultivar Pondus, Polish cultivar Radius and French cultivar Magali. The second cluster included Argentinian cultivar Barbara and two subclusters: (i) American cultivars Blazer XL and Magnum V-Wet and Swedish cultivar Sverre, and (ii) Croatian cultivars Vuka and OS-93, Italian cultivar Classe, Argentinian cultivar Victoria and French cultivar Europe. Most of the genetic variability estimated by AMOVA was attributed to variation among

TABLE 2

The number and percentage of polymorphic RAPD bands observed in each cultivar/population using the six primers.

Cultivar	Number of polymorphic bands	Percentage of polymorphic bands (%)
PONDUS	63	87.50
PRIME	67	89.33
GENESIS	71	89.87
MAGALI	63	86.30
MARON	52	83.87
RADIUS	59	85.50
BARBARA	69	86.25
VICTORIA	63	86.30
SVERRE	64	88.88
MAGNUM V-Wet	74	90.24
BLAZER XL	60	89.55
EUROPE	64	88.88
CLASSE	61	85.91
VUKA	61	87.14
OS-93	60	85.71
Average	63	87.41

individuals within cultivars (91.86%), and only 8.14% was found between cultivars (Table 4).

The average distance among the investigated alfalfa cultivars/population based upon the morphological data matrix was 0.15 (Table 3). The smallest distance was observed between Australian cultivar Genesis and Argentinian

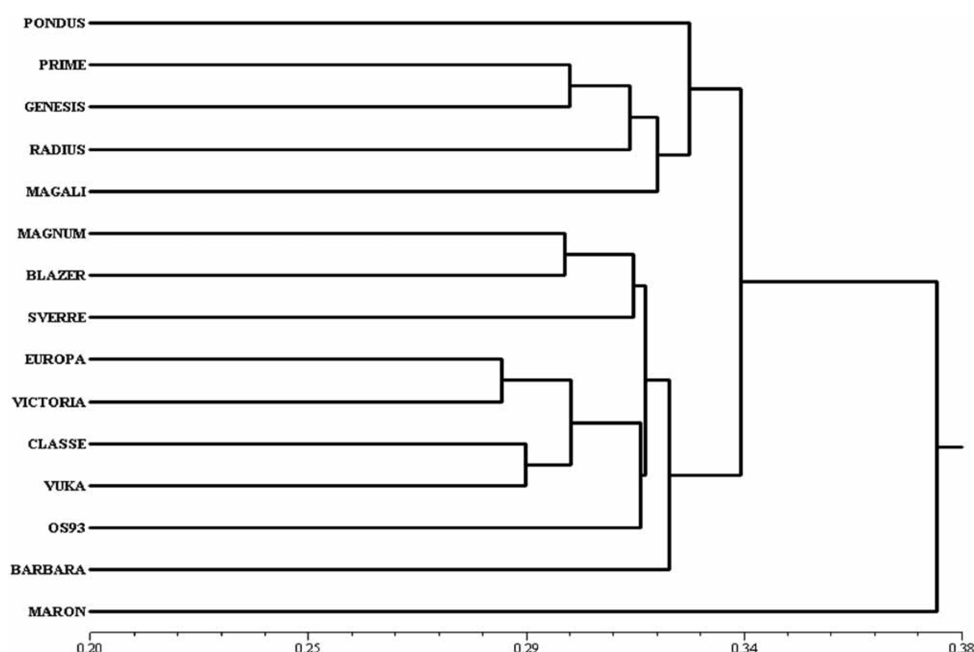

Figure 2. *Association among alfalfa cultivars revealed by cluster analysis (UPGMA) based on modified Rogers distance (MRD) calculated from 90 RAPD markers.*

cultivar Barbara (0.06), and the largest was (0.30) among Maron population (*M. falcata*) and *Medicago sativa* cultivars (Italian cultivar Classe, French cultivar Europe, Croatian cultivar OS-93 and Australian cultivar Prime). The cluster analysis based on morphological distances among the alfalfa cultivars/population (dendrogram not shown) revealed great differences between Maron population (*M. falcata*), which formed separate branch, and all 14 investigated alfalfa cultivars. Cultivars were not clustered by their geographical origin. The Mantel Z test statistics showed no significant correlation (r = 0.10) of matrices based on RAPD markers and morphological characters.

DISCUSSION

Since only a wide genetic base gives the opportunity to select genotypes with a trait of interest, it is essential to understand the extent and distribution of genetic variation. This information is particularly important in alfalfa which is an allogamous and self-incompatible species susceptible to severe inbreeding depression. Decreased heterozygosity and heterogeneity of populations will decrease vigor and productivity.

Data on the genetic diversity of alfalfa cultivars/population with different geographical origin are presented in this study. Based on results of previous investigations on genetic relationships of allogamous forage crop species by RAPD markers *(12, 15, 26)*, 20 individuals were sampled per each cultivar/population. The six primers used in this study amplified 90 polymorphic bands across all alfalfa cultivars/population, with an average of 15 polymorphic bands per primer. This is in agreement with the number of RAPD bands used by Musial *et al. (7)* who analyzed genetic diversity within Australian alfalfa cultivars, and slightly higher than the number of RAPD markers used to investigate white clover collection and cultivars *(13)* and *Dactylis glomerata* populations *(16)*. The proportion of polymorphic bands observed in Maron population was considerably lower in comparison with other investigated cultivars. This was probably a consequence of geographical isolation which limited the extent of gene flow from cultivated *Medicago* species. The diverse proportion of polymorphic bands observed across *M. sativa* cultivars was probably a reflection of differences in the amount and type of germplasm used to develop these cultivars. In addition, most of the investigated cultivars are synthetic populations and other factors, such as the number of parental plants included in their development or a number of selected plants or previous selection for a specific trait affected the level of polymorphism. RAPD-PCR analysis revealed two specific bands for the Maron population and one for the Radius cultivar. Results indicated that the applied primers may be used in the identification of these materials. Identification of band 850 bp (generated by primer RAPD 4) in plants of Maron population and Radius cultivar may by a explained by significant share of *falcata* genome in Radius cultivar (interspecies hybrid).

The Maron population (*M. falcata*) was the most distant from (Table 3) the other alfalfa cultivars analyzed. The dendrogram based on UPGMA cluster analysis also showed that the Maron population clustered separately from all the other alfalfa cultivars, which is congruent with the results of Musial *et al. (7)*, Segovia-Lerma *et al. (10)* and Maureira *et al*. *(4)*, who used molecular markers to study genetic diversity of *Medicago sativa* germplasm. The results indicated that Maron population is genetically divergent from the investigated alfalfa cultivars, and

TABLE 3

Genetic distances among alfalfa cultivars/population calculated by RAPD data (lower triangle) and morphological characteristics (upper triangle) data.

	BARBARA	BLAZER	CLASSE	EUROPE	GENESIS	MAGALI	MAGNUM	MARON	OS93	PONDUS	PRIME	RADIUS	SVERRE	VICTORIA	VUKA
BARBARA	–	0.14	0.12	0.16	0.06	0.14	0.14	0.28	0.17	0.17	0.12	0.20	0.13	0.13	0.09
BLAZER	0.32	–	0.18	0.12	0.16	0.16	0.14	0.26	0.09	0.07	0.14	0.14	0.09	0.10	0.07
CLASSE	0.33	0.33	–	0.18	0.14	0.13	0.16	0.30	0.20	0.23	0.12	0.21	0.18	0.14	0.15
EUROPE	0.30	0.29	0.31	–	0.19	0.13	0.15	0.30	0.10	0.14	0.13	0.15	0.12	0.15	0.13
GENESIS	0.32	0.32	0.33	0.31	–	0.15	0.13	0.26	0.18	0.17	0.15	0.20	0.13	0.14	0.11
MAGALI	0.33	0.36	0.35	0.33	0.31	–	0.14	0.29	0.15	0.18	0.11	0.16	0.14	0.14	0.15
MAGNUM	0.30	0.29	0.33	0.29	0.32	0.34	–	0.29	0.14	0.13	0.16	0.14	0.09	0.12	0.12
MARON	0.40	0.37	0.38	0.37	0.38	0.38	0.38	–	0.30	0.28	0.30	0.29	0.28	0.27	0.28
OS93	0.34	0.32	0.30	0.32	0.33	0.35	0.32	0.38	–	0.08	0.14	0.10	0.08	0.14	0.12
PONDUS	0.33	0.34	0.32	0.33	0.31	0.33	0.34	0.38	0.34	–	0.17	0.10	0.07	0.11	0.10
PRIME	0.34	0.34	0.33	0.32	0.29	0.32	0.33	0.36	0.34	0.32	–	0.20	0.14	0.15	0.13
RADIUS	0.34	0.34	0.34	0.33	0.31	0.31	0.35	0.35	0.34	0.33	0.31	–	0.10	0.15	0.16
SVERRE	0.31	0.31	0.33	0.30	0.34	0.34	0.31	0.38	0.31	0.33	0.34	0.35	–	0.11	0.08
VICTORIA	0.30	0.30	0.30	0.28	0.32	0.34	0.30	0.37	0.32	0.35	0.32	0.32	0.30	-	0.09
VUKA	0.33	0.31	0.29	0.29	0.33	0.34	0.32	0.38	0.30	0.34	0.33	0.32	0.31	0.29	-

therefore a potential source of novel, favorable alleles that could be used to improve alfalfa cultivars. Kidwell *et al. (27)* also reported significant positive correlations between genetic diversity of parental alfalfa genotypes and the forage yield of their single-cross progenies. Most European *M. sativa* cultivars (with exception of Pondus and Magali) clustered together, which suggests that they have common genetic background. The clustering of USA cultivars Blazer XL and Magnum V-Wet with European cultivars could be explained by common ancestry (Flemish – type germplasm used to develop these cultivars – The North American Alfalfa Improvement Conference, http://www.naaic.org/). Probably from the same reason, Argentinian cultivars Barbara and Victoria (pedigree data known) were clustered in the same group. The cluster analysis showed that the Australian cultivars Genesis and Prime and the Polish cultivar Radius (*M. media*) are distanced from the most European cultivars. Thus, they offer a new source of genetic diversity for the European *M. sativa* gene pool. The AMOVA revealed a higher distribution of genetic variation within cultivars. This is in agreement with the partitioning of variation observed among Italian ecotypes and cultivars alfalfa based on RAPD bands and SSR alleles *(26)*. A similar result was obtained in analysis of genetic variation of other allogamous forage species. Kolliker *et al. (14)* found in *Dactylis glomerata* about 85.1% of variation among genotypes within cultivar. Vieira *et al. (28)* reported that 98% of total diversity in annual ryegrasses was intra-populational. The AMOVA of diverse red clover breeding population and

TABLE 4

Analysis of molecular variance (AMOVA) of 15 alfalfa cultivars/population.

Source of variation	df	SSD*	Variance component	Percentage of variance
Among cultivars	14	5.374	0.0124	8.14
Within cultivars	282	39.306	0.1394	91.86

*Sum of squared deviations

cultivars indicated that these materials had the largest proportion of variation (80.4%) within population *(11)*.

Genetic diversity based on morphological data was high between Maron population (*M. falcata*) and all *M. sativa* cultivars, which is in accordance with previous analyses of alfalfa diversity using agronomic and morphological characteristics *(29)*. The UPGMA dendrogram based on morphological characters (graph not shown) was unable to clearly differentiate *M. sativa* cultivars with different geographical origins. There are several possible explanations for this: (i) the small number of morphological characters and plants per cultivars included in investigation (ii) the influence of environmental conditions that prevented recognition of considerable variation (iii) bias in description of morphological characters. Dehghan-Shoar *et al. (30)* also reported about insufficient usefulness of plant morphological characters in identification and discrimination of Iranian and New Zealand alfalfa cultivars. In the present study, Mantels test did not confirm the correlation between molecular and morpho-

logical distances. The result suggests that the two marker systems give different estimates of genetic relations among cultivars. This is in agreement with studies of other authors who used morphological characters and molecular RAPD markers *(31-33)*.

The results of this study indicate that RAPD analysis could be successfully used for the estimation of genetic diversity among alfalfa cultivars. Also, they showed that Maron population (*M. falcata*), Australian *M. sativa* cultivars Prime and Genesis and Polish cultivar Radius (*M. media*) are genetically diverse from most of the evaluated European alfalfa (*M. sativa*) cultivars. This genetic distinctiveness suggests that these sources have novel alleles which may, by introducing them to European alfalfa (*Medicago sativa*) gene pools, produce a positive heterotic response in a breeding programmes.

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