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

T. Cholastova, D. Knotova

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 milion 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.

Tereza Cholastova, Agricultural Research, Ltd., Department of Plant Physiology and Genetics, Troubsko, Czech Republic (corresponding author to provide e-mail: cholastova@vupt.cz)

Daniela Knotova, Research Institute for Fodder Crops, Ltd., Troubsko, Czech Republic (e-mail: knotova@vupt.cz)

On the other hand, DNA-based molecular markers allow a more precise and environment-independent way of studing 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 repaets (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 $10m^2$, 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. pastbiščnaja	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 µl total volume containing 1x reaction buffer, 100 µM dNTPs, 10 µM of each SSR primer, 1.5 mM $MgCl_2$, 2 µl 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.uc.uk/rod/rod.html).

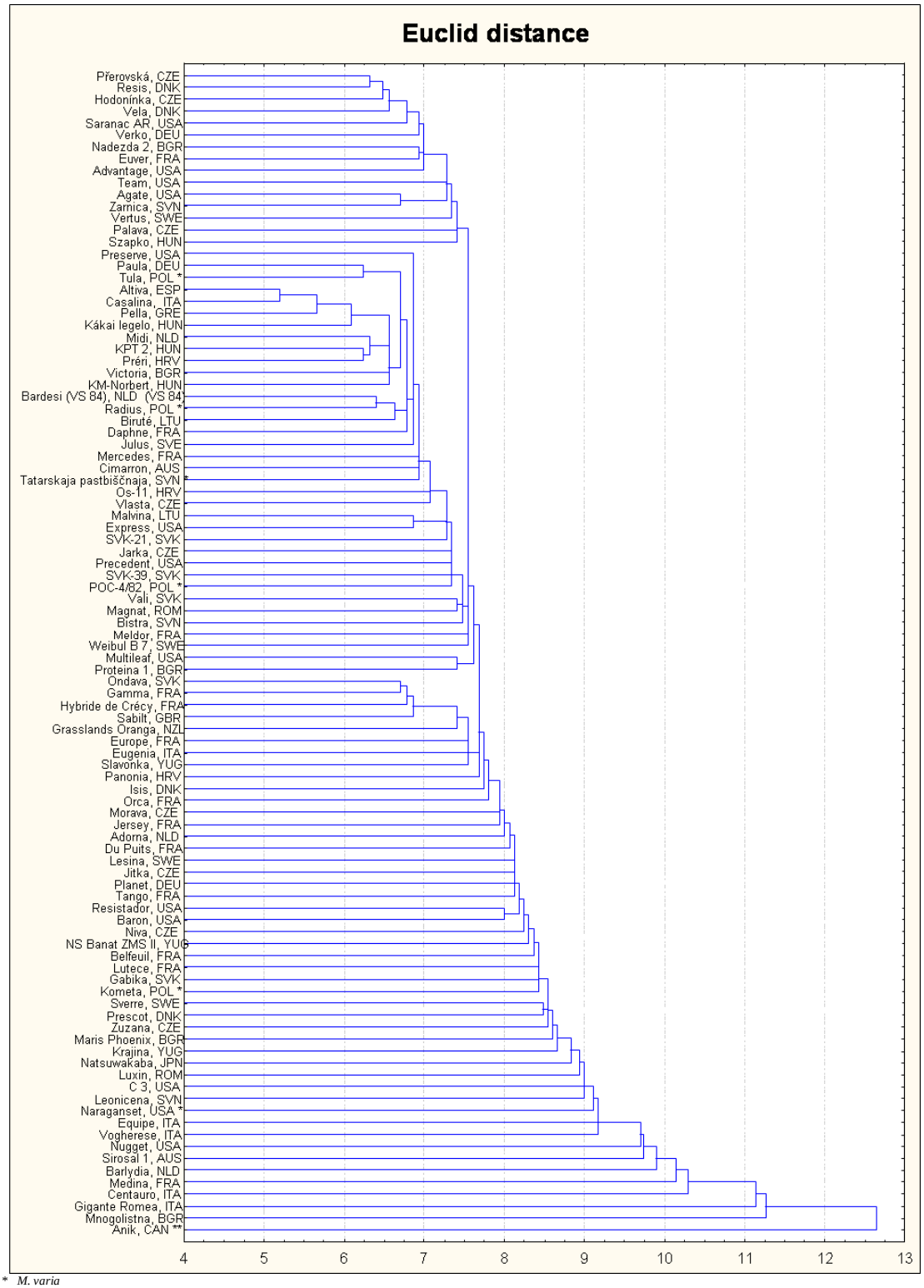
Cluster analysis was computed by help of the programme Statistica 7.1 [16] (StatSoft, Inc. 1984 – 2005). For calculation the Euclidean distances, Unweighted Pair Group Method with Arithmetic Means (UPGMA) was used.

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 polymorpism 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_e value for all three used SSR markers was very high (> 0.90) (Table II).

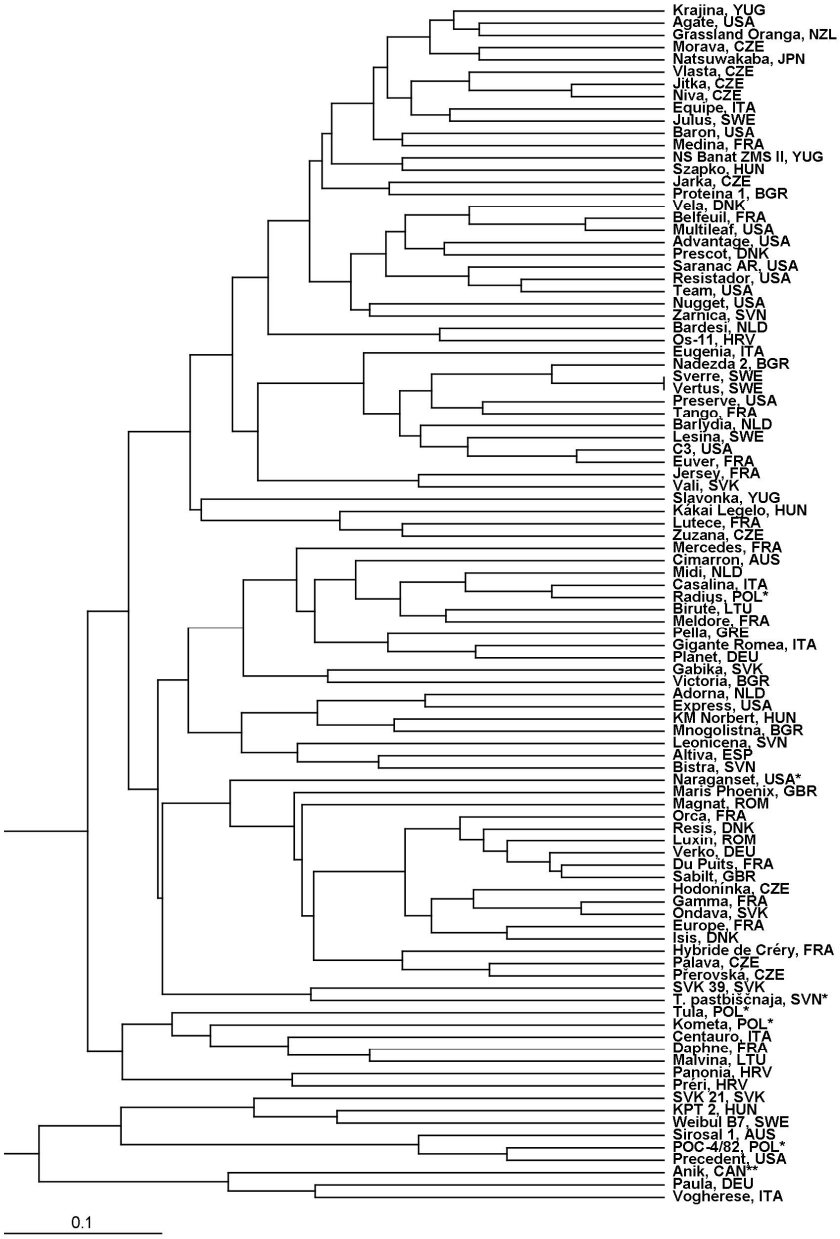
TABLE II
NUCLEOTIDE SEQUENCES OF 3 POLYMORPHIC PRIMERS AND PIC AND HE VALUES IN SSR ANALYSIS

Locus	Core motif	Forward primer	Reverse primer	PIC	H
AFctt1 (D)	$(CTT)_9(CAA)_3$	cccatcatcaacattttca	ttgtggattggaacgagt	**0.9121**	**0.9178**
MTIC297 (T)	$(TAC)_5$	ctaagctttggccatgtatc	tgaaatgagtttgactgagg	**0.901**	**0.9082**
B21E13 (J)	$(GA)_{13}$	gccgatggtactaatgtagg	aaatcttgcttgcttctcag	**0.9163**	**0.9214**

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 discance 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. pastbiščnaja (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 depedence of morphological traits, as well as uncertainty ragarding 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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