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RAPD polymorphism in the prebreeding material for cultivation of synthetic variations of lucerne (*Medicago sativa* L.)

Research Article

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Abstract: Genetic diversity between synthetic cultivars (Syn5, Syn7), inbred lines (D_3, D_5, $E_{1/2}$, $G_{1/1}$, $G_{1/2}$) and ecotypes (E16, E51, E182, E231) of lucerne (*Medicago sativa* L.) was studied using the RAPD-PCR method. The plants differed in the efficiency of seed set and in the yield of green mass. The ecotypes E182 and E231 and the synthetic population Syn5 showed the highest fertility. Additionally, Syn5 also showed the highest efficiency of seed set and the yield of green mass. Among the inbred lines, D3 was characterised by the highest yield of green mass and E1/2 by the highest fertility. An optimal combination of yield and biomass was observed for the synthetic population Syn5, obtained by crossing the lines D_3, D_5 and $G_{1/1}$, as demonstrated using comparative analysis. A total of 338 polymorphic products were generated using 20 arbitrary primers. Cluster analysis using the *Unweighted-Pair Group Method with Arithmetic Mean* (UPGMA) in the Molecular image Gel Doc™ XR (Bio-Rad) software based on the Dice's coefficient of genetic similarity showed a division of the studied forms into two groups based on genetic similarity. The ecotype E16 formed one of the groups whereas all of the other ecotypes observed in this study clustered into the second group. A high level of polymorphism among the studied lucerne forms was detected indicating an interesting gene pool awaiting future exploration. Analysis of variance also supported a high diversity among the studied forms. This study provides insightful information into the heterosis effect of synthetic populations or hybrids of F1 lucerine by providing correlations between the genetic background of the inbred lines and their ability to produce a specific yield.

Keywords: *Medicago sativa L.* • *Prebreeding material* • *RAPD* • *Polymorphism*

1. Introduction

With about 32 million hectares cultivated globally, the *Medicago* is one of the most important forage crops [1]. The plant in its current form is believed to have originated in the Caucasus region, which today extends over northeastern Turkey, Turkmenistan and northwestern Iran [2]. Not only is it the oldest cultivated plant, but also the oldest wild crop in this area. Due to its genetic structure, cultivation of alfalfa, which is another name for lucerne, is a rather complex process. Cultivated alfalfa (*Medicago sativa* L.) is autotetraploid (2n=4x=32), allogamous and seed propagated [3].

The synthetics alfalfa cultivars are developed by successively intercrossing selected plants and increasing the seed yield in 2-3 generations. For alfalfa breeders a desirable goal in these cultivars is capturing heterosis. In breeding of synthetic cultivars, there is an inbreeding stage. Unfortunately, alfalfa suffers severe inbreeding depression upon repeated selfing. Severe inbreeding depression results from the high level of deleterious recessive alleles carried by alfalfa

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plants. Autotetraploid alfalfa displays a certain level of heterosis in certain types of crossing. The level of inbreeding depression depends on the genetic loading of selfing plants. In selfing plants, deleterious or lethal recessive alleles, which were masked by favorable dominant ones, are exposed and mostly eliminated [4,5]. The low number of seeds that the plant produces in Polish climatic conditions is a cause decreasing alfalfa production, which is not very profitable. Therefore improving the seed yield in the cultivated forms of alfalfa is an essential objective for Polish breeders [6,7].

There are many sources of desirable genes, which, when incorporated into new lucerne cultivars, would improve their seed yield. One such a source is germplasm taken from natural populations (landraces) that can be used as a component in crossing with a cultivar being improved. Landraces are genetically dynamic with variations existing among sites and populations as well as within sites and populations [5,8]. The individuals of a cross-pollinated landrace, although phenotypically similar, are highly heterozygous and genotypically different [3,5]. Adaptive genes flow from landraces to cultivars is possible. The hybrids obtained in this way must be subjected to selection by the breeder through sowing seeds collected from the plants that have the highest seed yield and a high yield of green matter. Another method of improving plant yield is to select, on the basis of general combining ability and specific combining ability, those inbred lines that are most promising for creation of synthetic populations [5,9-12]. Therefore, in breeding approaches which aim at developing new synthetic cultivars, a number of superior single plants must be collected through intermating in crossing blocks. The collected plants must be genetically identified through an evaluation of genetic diversity. One method of doing this is to use molecular markers [13,14].

Different molecular marker types that have been used to assess genetic diversity of alfalfa include among others: random amplified polymorphic DNA (RAPD) [15], amplified fragment length polymorphism (AFLP) [16], restriction fragment length polymorphism (RFLP) [17], simple sequence repeat (SSR) [18], inter-simple sequence repeat (ISSR) [1], and sequence related amplified polymorphisms (SRAP) [19]. Random amplified polymorphic DNA (RAPD) is a quick and simple technique that continues to be used extensively. The main advantage for using RAPD in designing a molecular marker is that no information on the target sequences in the genome is required.

This study characterizes the genetic variability of the populations used for creating new synthetic cultivars, using RAPD method for quick and precise molecular analysis. Introducing synthetic populations of alfalfa enhances the heterosis effect. Examining the relationship between the inbred lines and synthetic populations allows for an identification of the genetic relationships between them. Hence, in analyzing the inbred lines, it is possible to select components that may be used for creating a synthetic population. In other words, the genetic analysis of the inbred lines allows for selecting components that may be used in creating synthetic populations.

2. Experimental Procedures

2.1 Materials

Two synthetic populations – Syn5 characterized by the highest yield of seeds and green mass and Syn7 with the lowest yield of seeds and green mass, six inbred lines D_3, D_5, $E_{1/2}$, $G_{1/1}$, $G_{1/2}$ and four landraces: E51 and E182 with the highest seed set and E16 and E231 with the lowest seed set were investigated. The synthetic population Syn5 was composed of the lines D_3, D_5 and $G_{1/1}$, and Syn7 consisted of D_5, $E_{1/2}$, $G_{1/1}$. The inbred lines were developed as described above [5].

The materials were analyzed during a 3-year field experiment conducted in four replications at the Experimental Station of University of Life Sciences in Poznań-Dłoń, Poland. The inbred lines in the F5 generation were derived from crosses between the following cultivars: Polish (Boja, Radius), French (Du Puits, Rezis), American (Vernal) and the landraces collected in Poznań region of Poland. The reasoning behind such a choice of starting genetic material was to do the crosses using material of the widest possible genetic origin. A total of 112 hybrids were derived from the inbred lines, out of which eight inbred lines with the highest Specific Combining Ability (SCA) were selected in the F5 generation. The seeds were sown in autumn on 10 m^2 plots in rows 20 cm apart (15 kg/ha). The yields of seeds were evaluated for synthetic and natural populations in the field with 10 m^2 plots, rows 25 cm apart (7 kg/ha).

The plants from the inbred lines were grown in a greenhouse, where they were isolated. Seeds from 30 individual plants were harvested and a mean value of each studied trait was calculated for a single average plant. An average seed yield, fertility and pod set values were also calculated for a single average plant as a mean value measured from 50 plants taken from the synthetic populations and landraces of alfalfa. Fertility was expressed as a ratio between the number of seeds in the pod and the number of ovules in the ovary. The number of ovules was counted under the binocular in all the plant materials, and ranged between 8 and 12. There was no correlation between the number of ovules and the number of seeds per pod.

The trait of pod set was evaluated as the number of flowers in inflorescence per the number of pods. The yield of green matter was evaluated on the basis of three cuttings of plants from the plot and it was expressed as the mean of dry matter in percentage. The percentage of dry matter makes it possible to calculate the yield of dry matter per ha, which sometimes can be a better indicator than the yield of green matter.

2.2 DNA extraction and amplification

For molecular analysis, DNA was extracted from approximately ten plants from each line. The plants were equally mixed (bulked). A DNA sample bulked from ten individuals is reported to provide a good representation of variations that exist in a particular cultivar or a particular population of lucerne as demonstrated using the RAPD markers [20,21].

DNA was extracted with an additional proteinase K treatment [22]. DNA concentration was quantified using a *Smart Spec*™ *Plus* (Bio-Rad) spectrophotometer. PCR-RAPD reactions were performed for 20 10-nucleotide Operon Technologies primers (Table 1). The amplification reactions were performed in volume 0.04 cm^3 containing: 600 ng DNA, 0.75 mM primer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 2.5 U *Taq* polymerase (Fermentas), 1x reaction buffer and water. Predenaturation was conducted for 12 minutes at 95°C, then in 45 cycles: for 1 minute at 94°C, for 1 minute at 34°C, and for 2 minutes at 72°C, and terminated for 5 minutes at 72°C. The PCR products were electrophoretically separated on 1.5% agarose gel in 1xTBE buffer. After electrophoresis, the gel was incubated in ethidium bromide (2.5 µg/ml) for 10 min. and photographed with GelDoc 1000 using the Quantity-one software (Bio-Rad). The gels were documented in the Bio-Rad gel documentation system and the molecular weight of the fragments was determined using the Quantity-one software (Bio-Rad). PCR-RAPD reactions were performed twice for each primer – individual DNA sample combinations and only reproductible bands were analyzed.

The molecular data was coded on binary basis and analysed using Molecular Image Gel Doc™ XR (Bio-Rad). Similarity index was estimated using the Dice's coefficient of similarity [23]. Cluster analyses were carried out on similarity estimates using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [24].

Name of primer	Primer sequence [5'-3']	Size range [bp]	Total number of products	No. of polymorphic products	P [%]
OPC-02	GTGAGGCGTC	94-662	6	1	16.66
OPC-04	CCGCATCTAC	112-913	7	4	57.14
OPC-05	GATGACCGCC	302-1979	7	5	71.4
OPC-06	GAACGGACTC	88-1581	6	2	33.,33
OPC-07	GTCCCGACGA	92-883	10	9	90
OPC-08	TGGACCGGTG	101-639	7	6	85.7
OPC-09	CTCACCGTCC	186-1934	8	6	75
OPC-11	AAAGCTGCGG	147-1222	9	9	100
OPC-13	AAGCCTCGTC	172-839	5	4	80
OPC-16	CACACTCCAG	166-741	5	3	60
OPP-06	GTGGGCTGAC	258-2261	21	21	100
OPP-07	GTCCATGCCA	463-1838	14	14	100
OPP-08	ACATCGCCCA	196- 2528	34	34	100
OPP-09	GTGGTCCGCA	223-1226	27	27	100
OPP-10	TCCCGCCTAC	81-476	12	12	100
OPP-11	AACGCGTCGG	85-3754	53	53	100
OPP-12	AAGGGCGAGT	154-1044	19	19	100
OPP-13	GGAGTGCCTC	225- 2390	32	32	100
OPP-15	GGAAGCCAAC	96-2569	41	41	100
OPP-18	GGCTTGGCCT	95-2611	36	36	100
Total		81-3754	359	338	83.46

Table 1. Primers employed with the number of RAPD markers obtained, the size of the fragments, and the percentage of polymorphic markers (P) for each primer.

2.3 Statistical analysis

Normality of distribution of the studied traits was tested using by the Shapiro-Wilk test [25]. The one-way analysis of variance [26] for statistical verification of significant differences between the analysed traits was performed. Least significant differences (LSD) for each trait were calculated. Homogeneous groups for the analysed traits were determined on the basis of least significant differences. The relationship between the analysed traits were estimated using correlation coefficients. Interrelations between the traits were presented as scatterplots [27]. The results were also analysed using multidimensional methods [28]. Multidimensional phenotypic diversification of lucerne forms was determined on the basis of the Mahalanobis distances [29]. The canonical variate analysis was applied for the multivariate assessment of 11 objects. The GenStat v. 7.1 statistical software was used for the data analysis [30].

3. Results

The results of analysis of variance indicated that the studied forms of alfalfa differed significantly in all of the studied traits (Table 2). As described previously [5], the highest fertility was found in ecotypes (landraces) E51 and E182 and in the synthetic population Syn-5. Population Syn-5 also produced the highest yield of seeds/plant. These traits were accompanied by a high pod set and a high yield of green mass. Lower values of these traits than Syn-5 and E51 and E182 were in the inbred lines. D_3 had the highest yield of seeds/plant among the analyzed lines, while $E_{1/2}$ was characterized by the highest fertility [5]. In contrast to the other inbred lines, Syn-5 consisted of lines D_3, D_5 and $G_{1/1}$, which gave the best specific combination expressed in the values assigned to the selected traits (Table 3).

E51 was characterised by the highest values in all of the traits. A large number of single-variation

Source of variation	Degrees of freedom	Analyzed traits				
		Fertility	Yield of seeds / plant	Effectiveness of pod set	Yield of green mass	Dry matter content
Replications	2	2.422	0.3512	2.817	2.164	0.1458
Genotypes	10	1442.671***	98.2242***	1418.898***	648.386***	13.4967***
Residual	20	1.447	0.4575	1.949	4.857	0.6588

Table 2. Means squares from analysis of variance for tested traits of 11 forms of alfalfa.

(model $y = \mu + bx + e$, where y is value of the observed trait – dependent variable, μ is general mean, x is genotype – independent variable, b is genotype effect and e is independent error)
** significant at $\alpha = 0.001$ level*

Alfalfa Forms	Analyzed traits				
	Fertility	Effectiveness of pod set	Yield of seeds /plant	Yield of green mass	Dry matter content
D_3	30.8h#	34.2ef	5.0def	38.5c	21.9cde
D_5	34.7g	45.1d	3.6f	33.8cde	20.4de
$E_{1/2}$	46.3e	30.8f	3.5f	31.2de	22.9bcd
E16	64.6c	65.8c	6.4d	49.3b	20.1e
E182	89.7a	91.2a	18.2a	69.1a	25.6a
E231	53.6d	71.7b	8.6c	54.8b	24.0abc
E51	88.5a	88.2a	19.0a	63.6a	25.7a
$G_{1/1}$	39.8f	35.2e	4.1ef	35.0cde	24.2abc
$G_{1/2}$	34.2g	46.3d	3.3f	29.1e	20.1e
Syn-5	72.0b	73.6b	11.4b	63.8a	24.9ab
Syn-7	35.3g	47.8d	6.2de	37.1cd	23.4abc
$LSD_{0.001}$	3.78	4.39	2.13	6.93	2.55

Table 3. Mean values, least significant differences and homogeneous groups for the analyzed traits of 11 forms of alfalfa.

 # – in columns means followed by the same letters are not significantly different

homogeneous groups was obtained, which indicates a high variation of genotypes (Table 3). Table 4 shows a correlation matrix for the traits. All the correlations were positive and statistically significant. The highest correlation coefficient was observed for the yield of green mass and for the effectiveness of pod set (r=0.938). Linear relations were observed for the majority of trait pairs (Figure 1). The only non-linear relation was observed between the efficiency of the pod set and dry mass content (Figure 1).

Table 5 shows Mahalanobis distances between the genotypes. The greatest similarity was observed between E51 and Syn-7 (Mahalanobis distance equals 2.75). The largest value of Mahalanobis distance (73.91) was obtained for $G_{1/1}$ and Syn-5 (Table 4). Figure 2 shows the distribution of the genotypes analysed in the space of the first two canonical variates. The first two canonical variates explained 97.61% of total variability. The genotypes were grouped based on the analysis of all the traits. Two groups of objects were distinguished: the first one includes E51, Syn-7 and E231, and the second one D_5, E182, $G_{1/1}$ and $G_{1/2}$ (Figure 2). The other genotypes make single-element groups. When testing lucerne using the RAPD markers, its variability is usually very high, which is probably due to allogamy of the species and the fact that alfalfa is tetraploid [31,32]. In this study, 20 random primers produced a total of 338 fragments, ranging from 81 bp to 3754 bp,

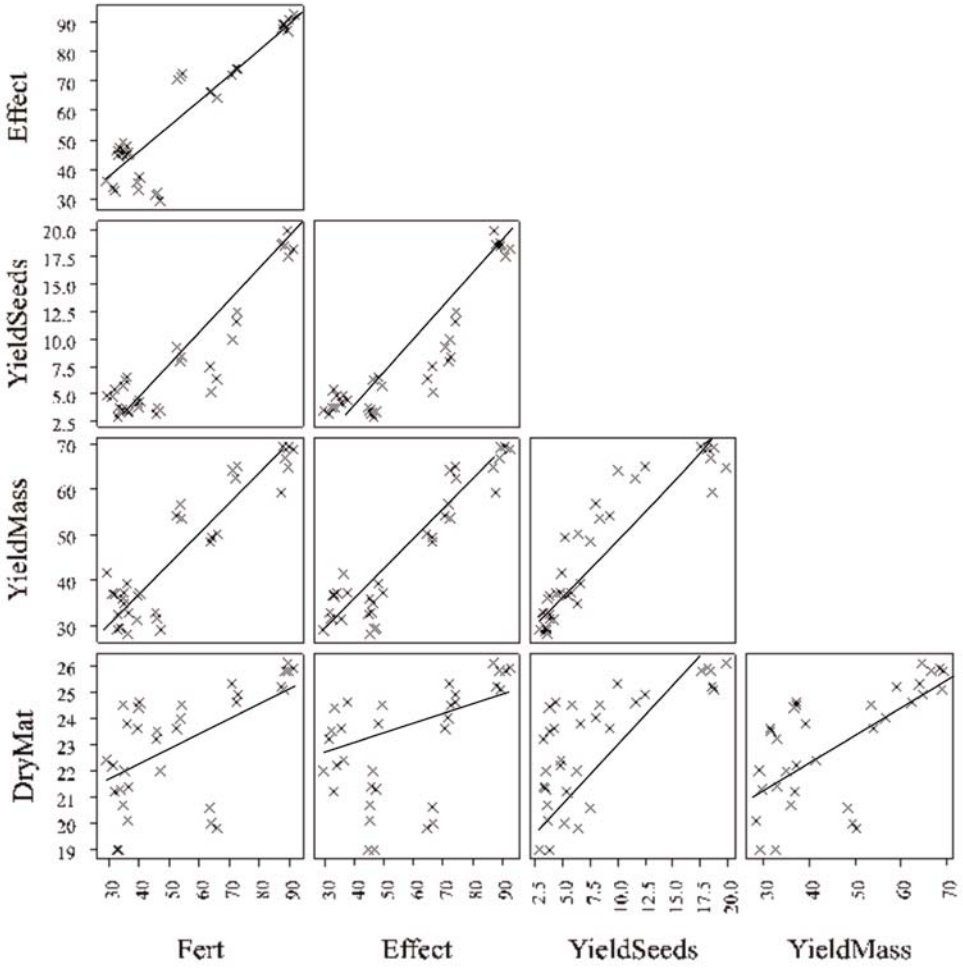

Figure 1. Scatterplot matrix for five alfalfa traits (Fert - fertility, Effect - effectiveness of pod set, YieldSeeds - yield of seeds/plant, YieldMass - yield of green mass, DryMat - % dry matter with a superposed line representing a linear relationship between traits. All correlation coefficients were positive and statistically significant: fertility - effectiveness of pod set, r=0.909; fertility - yield of seeds per plant, r=0.921; fertility – yield of green mass, r=0.920; fertility - % dry matter, r=0.640; effectiveness of pod set - yield of seeds per plant, r=0.907; effectiveness of pod set - yield of green mass, r=0.938; effectiveness of pod set - % dry matter, r=0.550; yield of seeds per plant - yield of green mass, r=0.914; yield of seeds per plant - content of dry matter, r=0.752; yield of green mass - % dry matter, r=0.688.

		1	2	3	4	5	6	7	8	9	10
Syn-5	1	0									
Syn-7	2	12.44	0								
D3	3	13.68	15.68	0							
D5	4	44.44	34.43	39.42	0						
G1/1	5	73.91	65.12	69.07	33.13	0					
E1/2	6	38.35	28.47	36.58	12.7	37.87	0				
G1/2	7	70.96	62.3	66.02	31.12	4.39	35.49	0			
E16	8	8.12	12.33	6.54	40.88	70.39	36.04	67.34	0		
E51	9	14.06	2.75	16.82	34.75	65.44	28.79	62.58	13.64	0	
E182	10	51.15	42.49	46.49	12.17	23.44	16.58	21.3	47.63	43.07	0
E231	11	11.65	5.96	16.23	35.6	64.56	28.21	61.57	11.62	6.95	42.23

Table 4. Mahalanobis distances between 11 forms of alfalfa.

	Syn5	Syn7	D3	D5	E1/2	G1/1	G1/2	E 16	E 51	E 182	E 231
Syn5	100.0										
Syn7	72.2	100.0									
D3	74.8	71.0	100.0								
D5	66.0	65.1	72.9	100.0							
E1/2	66.7	75.7	70.6	61.6	100.0						
G1/1	61.3	63.0	70.9	74.4	64.7	100.0					
G1/2	67.4	79.0	70.6	70.5	75.8	78.8	100.0				
E 16	30.7	31.4	32.4	35.0	35.3	30.1	33.6	100.0			
E 51	62.0	65.2	63.1	49.4	58.0	58.4	68.6	38.7	100.0		
E 182	58.7	57.3	58.6	61.0	53.8	60.9	56.1	25.9	56.6	100.0	
E 231	58.9	51.1	57.7	56.0	58.1	49.8	48.0	36.0	49.1	59.3	100.0

Table 5. Dice' similarity matrix of 11 forms of alfalfa.

all of which were polymorphic (Table 1). Primer OPP-11 had the highest number of polymorphic PCR bands (53), while primer OPC-02 showed the lowest number of polymorphic bands (1) (Table 1). Total percentage of polymorphism from all the primers was 83,46 (Table 1).

The genetic similarity between the studied lucerne forms ranged from 25.9 to 79.9 (Table 5). The highest similarity was observed between the inbred lines Syn7 and $G_{1/2}$. The genetic resemblance between the landraces E51 and E182 and the inbred lines was very low, contrary between the inbred lines. These two landraces, characterized by the highest seed yield per plant were distant from the synthetic populations Syn5 and Syn7. The dendrogram (Figure 3) presents the division of 11 alfalfa forms into two groups. The first group includes only the ecotype E16. The second group is split into two subgroups: the first subgroup includes the ecotypes E182 and E231, while the second one the remaining forms of alfalfa.

4. Discussion

Insufficient fertility of lucerne cultivars is a serious problem affecting cultivation of lucerne in Poland. Hence, years ago, a research/breeding programme was launched at the Chair of Genetics and Plant Breeding of University of Life Sciences, Poznań, Poland. The programme was aimed at improving this very trait. However, the plant material used in those studies was never analyzed in terms of its genetic diversification, which is rather important for the development of synthetic variations.

The genetic distance or similarity of the pre-breeding material is very important for creating new synthetic alfalfa populations that would express heterosis vigour. The three high yielding landraces were separated from the rest of entries. Similar results were obtained by analyzing mtDNA specific sequences in most of these lines [5]. The highest resemblance was observed between Syn-7 and the line $G_{1/2}$, and between $G_{1/2}$ and line $G_{1/1}$. The possible way to improving combining ability in synthetic populations of alfalfa is combining and

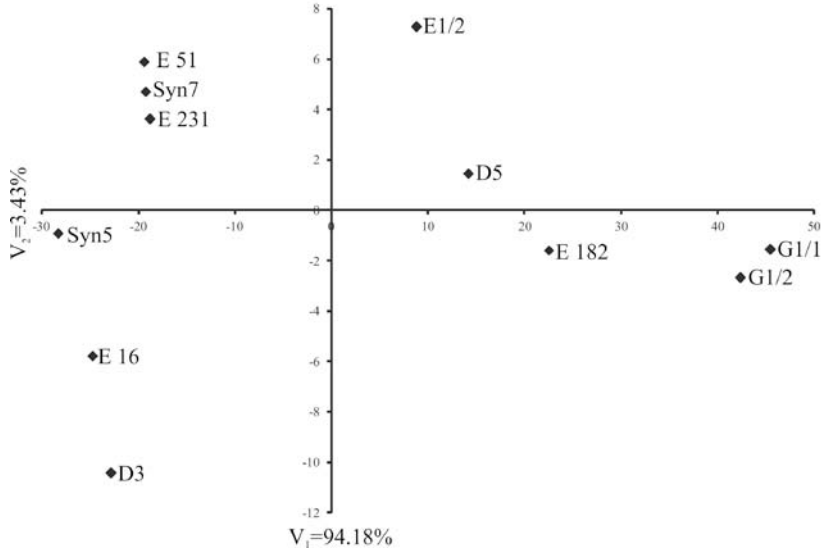

Figure 2. Distribution of 11 forms of alfalfa in the space of two first canonical variates for phenotypic traits (fertility, yield of seeds per plant, effectiveness of pod set, yield of green mass and dry matter content).

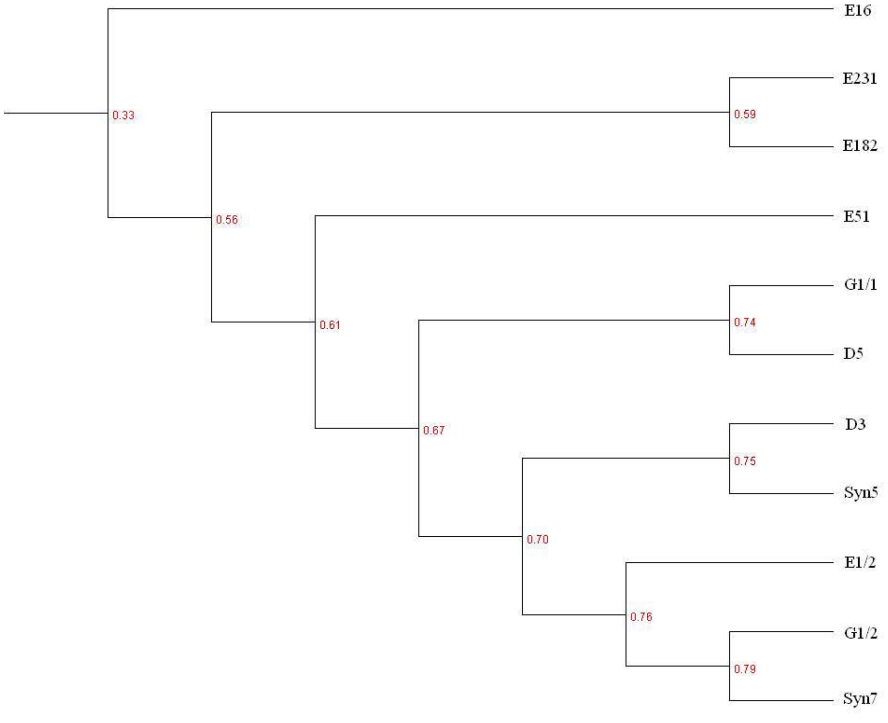

Figure 3. Dendrogram presenting genetic similarity of 11 forms of alfalfa based on the Dice Coefficient and constructed by UPGMA (Diversity Database; Bio-Rad).

enriching different alleles (complementing the alleles of inbred lines) in a single population. The source of these alleles are the landraces which were characterized by a very high seed yield and a high yield of green mass.

Although the RAPD method has many advantages, it also has a number of limitations. For example, it generates only dominant markers, which prevents detection of heterozygotic individuals or the necessity of preselection of starters/primers that would allow to obtain stable and clear electrophoreograms [33]. Moreover, obtaining products of the same length from the studied individuals does not always mean that the organisms have a homologous fragment of DNA. Single bands observed in the gel may in fact be composed of a few products of amplification [34]. Yet another problem is the repeating of band patterns. The band patterns are influenced by variations in the concentration of the matrix DNA, content of magnesium cations Mg^{2+}, changes in the concentration of polymerase *Taq* and the source of its origin, and a fixed temperature of denaturation [35,36]. How important it is to ensure exactly the same conditions of amplification? This may be best illustrated by the fact that different laboratories often obtain products of different lengths despite rigorously maintaining the same parameters of amplification [37]. All of the above factors have significant influence on the analysis of lucerne using the RAPD markers.

Previous studies using PCR-RAPD drew attention to the fact that only a small amount of products was obtained [20,31]. The average number of products generated using 10 primers was 5 and 3 to 8, respectively. A similar average number of products (3.7) was obtained in this study.

The usefulness of RAPD markers for genetic profiling depends on a few factors. The first one is the repeatability of the results of amplification. But the difficulties reported by many authors [38-41] did not pertain to the research presented in this work. After adjustment of the method, the same thermal profile was used for amplification with all the primers. Another factor is a correct choice of starters generating a relatively large number of polymorphous *loci*. The oligonucleotides primers used in this study generated high polymorphism (338 polymorphic bands), which allowed to determine the genetic distance between the synthetic variations, the inbred lines and the ecotypes of lucerne.

Higher levels of genetic diversity in alfalfa were reported using different marker systems, such as RAPD and ISSR [1,42], SSR [18] and mitochondrial DNA [5]. The multi-trait characterization of the lucerne varieties studied in the coordinate system of the first two canonical variables is reliable. These variables account for 97.61% of the total variation. The canonical variate analysis is widely used in the genetic and cultivation studies [43-47].

Quantification of genetic variations of important morpho-agronomic traits is essential in breeding programmes because it reveals the genetic structure of population. The wide diversity of the morpho-agronomic traits observed in this study is promising for selecting superior individuals for further breeding work. Certain forms of alfalfa in this study show the greatest multidimensional variation estimated on the basis of Mahalanobis distance, which might be an interesting subject for further studies on the cultivation of lucerne.

The results obtained in the study indicate a possibility that the landraces and the inbred lines, which are grouped into different groups of similarity, could become a source of new genotypes. Such genotypes might be used to create new and synthetic cultivars of alfalfa with a high seed yield. Furthermore, the results also indicate that the RAPD markers may be a useful tool for the investigating the genetic differentiation of alfalfa.

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