

BRIEF COMMUNICATION

Genetic relatedness in different *Medicago sativa* lines based on polymorphism of mitochondrial DNA specific sequences

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Abstract

Two synthetic populations (Syn-5 and Syn-7), four inbred lines and four landraces of alfalfa (*Medicago sativa* L.) were studied for genetic relatedness. These forms were characterized by different amount of seeds set and green mass yield. Two primer pairs of specific nucleotide sequences of mitochondrial DNA isolated from soybean were used. The mtDNA revealed the existence of significant polymorphism among the investigated forms of alfalfa. The genetic similarity (Dice coefficient) among studied forms of alfalfa ranged from 20.1 to 96.1. The greatest resemblances were noticed between D₅ inbred line and the population of Syn-5. The lowest resemblances were noticed between Syn-7 and E_{1/2}. The UPGMA dendrogram split investigated forms of alfalfa into two groups: first group include three landraces, the second consist of the rest analyzed forms. There are two landraces distinct with the highest seed set and yield of green mass: one in the first group, another one in the second group.

Additional key words: alfalfa, green mass yield, mtDNA, seed yield, UPGMA

Cultivated alfalfa (*Medicago sativa* L.) is autotetraploid ($2n=4x=32$) and allogamous. Alfalfa is seed propagated and majority of the alfalfa cultivars are synthetics, developed by successively intercrossing selected plants and increasing seed through 2 - 3 generations. Capturing heterosis in these cultivars is a desirable goal for alfalfa breeders. In the breeding of synthetic cultivars, there is an inbreeding stage. Rare plants expressing the trait or traits are chosen from the initial population and they are polycrossed. Then selection is carried out on that population and the selected plants are self-pollinated. Autotetraploid alfalfa suffers severe inbreeding depression upon repeated selfing and displays some level of heterosis with specific types of crossing. The level of inbreeding depression depends on genetic loading of selfing plants. In selfing plants, deleterious or lethal recessive alleles which were masked by favourable dominant ones will be exposed, and majority of them will be eliminated (Kimbeng and Bingham 1998). There are many sources of desirable genes which can be incorporated into new alfalfa cultivars. One of them is using of germplasm of natural populations (landraces) which are balanced with the environmental conditions

and pathogens. They are genetically dynamic with variations among sites and populations and within sites and populations (Harlan 1975). Although phenotypically similar, the individuals of cross-pollinated landrace are highly heterozygous and genotypically different (Falcinelli *et al.* 1994). Adaptive genes flow from landraces to cultivars are possible and the obtained hybrids must be subjected to selection by breeder through practice of sowing seeds collected from the plants with the highest seed yield and high yield of green matter as well. Another way of improvement of plant yield is to select the best inbred lines on the basis of general combining ability and specific combining ability for the creation of the most promising synthetic populations (Muller *et al.* 2001, 2003, Maureira *et al.* 2004). Therefore, breeding approaches aiming to develop new synthetic cultivars have to collect a number of superior selected single plants intermating in crossing block. The collected plants must be genetically identified through evaluation of genetic diversity. One way to do this is using molecular markers (*e.g.* Falahati-Anbaran *et al.* 2007).

There are many different molecular markers which

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Abbreviations: PCR - polymerase chain reaction; UPGMA - unweighted pair group method with arithmetic averages.

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can be applied for describing genetic diversity of investigated populations. One of them could be highly polymorphic specific nucleotide sequences of mitochondrial DNA (Campbell and Buchan 2002, Muller *et al.* 2006). The mitochondrial genome exhibits extraordinary variation in size, structure and replication. Use of this ancient and highly conserved variation in phylogenetic studies has become quite popular (Gray *et al.* 1998), because of its strictly maternal inheritance pattern (Forsthoefel *et al.* 1992).

In this study, genetic diversity of plant material involved in breeding program of alfalfa based on specific nucleotide sequences of mitochondrial DNA is presented. The aim is to determine the genetic similarity of landraces, inbred lines and synthetic populations which presented different pod set and seed set using specific mtDNA sequences.

Two synthetic populations (Syn-5 characterized by the highest yield of seeds and green mass and Syn-7 with the lowest yields of seeds and green mass), four inbred lines (D₅, D₃, E_{1/2}, G_{1/1}), and four landraces (E51 and E182 with the highest seed set and E16 and E231 with the lowest seed set) were investigated. The synthesis Syn-5 was composed of lines D₃, D₅ and G_{1/1}, and the Syn-7 consisted of D₅, E_{1/2}, G_{1/1}. The inbred lines were developed as follows: the individual plants with the highest pod set and seed set were selected from bulk populations of alfalfa and self pollinated for four-five generations. As a result, 56 lines were selected and they were tested on the basis of general (poly-cross) and specific combining ability. Based on the obtained results finally five inbred lines were chosen. In the area neighbouring the city of Poznań (Poland), landraces of highly fertile alfalfa occurred. They were sources for the development of four populations named: E51, E182, E16 and E231.

In years 2004 - 2006, field experiments were performed using the system of complete random blocks in three replications to evaluate yield of fresh matter for synthetic and natural populations. Plants of inbred lines were grown in a greenhouse, where they were isolated and selfed and seeds were harvested from each of 30 individual plants. Yield of seeds, fertility and pod set were calculated for single plants as a mean value measured from 50 plants. The yield of green matter was evaluated on the basis of three cuttings of plants from the plot.

DNA was extracted from young leaves, collected from all *M. sativa* seedlings using a procedure with proteinase K (Maniatis *et al.* 1982). DNA concentration was quantified with a *Smart SpecTM Plus* (Bio-Rad, Hercules, USA) spectrophotometer. Because no sequences were available for the alfalfa mtDNA genome, primers developed from sequencing the soybean (*Glycine max* L.) mtDNA genome were used (Campbell and Buchan 2002).

Two primer pairs which maximized polymorphisms were used: SF3 (TCC AAA TTT CCC ATC CAT CA) and SR1041 (TGA GCC CAA ACA AGA AAT CC); and

SR1041 with the forward primer SF6806 (TGG AAA CGG AGT TTT GCG AA). SF3 and SR1041 prime a region of cytochrome oxidase subunit I; SF6806 is within mitochondrial ribosomal protein subunit L5. The volume of the reaction mixture was 0.04 cm³. The reaction mixture was composed of 100 ng DNA, 1.5 μM primer, 150 μM dNTP, 1.5 mM MgCl₂, 1U *Taq* polymerase (*Fermentas*, Lithuania), 1× reaction buffer and water. Predenaturation was conducted for 3 min at 95 °C, for 30 s at 95 °C, for 1 min at 53 °C and for 2 min at 72 °C through 49 cycles and terminated for 10 min at 72 °C. The PCR products were electrophoretically separated on 1 % agarose gel in 1× TBE buffer. After electrophoresis, the gel was incubated in ethidium bromide (2.5 μg cm⁻³) for 10 min and photographed with *GelDoc 1000* using the *Quantity-one* software (*Bio-Rad*).

The analysis of variance for yield characteristics was conducted. The gels were documented in the *Bio-Rad* gel documentation system and the molecular mass of the fragments was determined using *Quantity-one* software. The images were transferred to the *CoTrix* software (available on the Internet at: <http://webdoc.gwdg.de/ebook/y/1999/whichmarker/csoftwar.htm>) for the transformation of the band positions into binary character matrix. The similarity matrix was computed using the Dice coefficient (Dice 1945). The dendrogram was constructed by unweighted pair group method with arithmetic averages (UPGMA; Diversity database; *Bio-Rad*).

The highest fertility was found for landraces E182 and E231 and for synthetic population Syn-5 which produced the highest yield of seeds per plant as well. These traits were accompanied by high pod set and high yield of green mass. Inbred lines were characterized by lower values of these traits and among the lines, D₃ had the highest yield of seeds per plant but the E_{1/2} line was characterized by the highest fertility (Table 1). The analysis of variance of five analyzed traits indicates statistically significant differences (at level $\alpha = 0.001$) among the alfalfa forms.

In order to clarify the genetic relationships among synthetic populations, inbred lines and landraces, we investigated the patterns of DNA variation of two mitochondrial genes, determined by PCR amplifications of two pairs of primers: SF3 and SR1041, and SF6806 and SR1041. The mitochondrial genes revealed the existence of significant polymorphism among the investigated forms of alfalfa (Fig. 1).

In this study, mtDNA variable nucleotide sequences, determined by two pairs of primers, of regions of cytochrome oxidase subunit I and from mitochondrial protein subunit L5, differentiated very well the investigated forms of alfalfa. This indicates that these mitochondrial genes or mtDNA sequences are very suitable for the determination of genetic differentiation of alfalfa. The same mitochondrial genes were used in molecular analysis of the genetic similarity of three species of *Medicago* (Campbell and Bauchan 2002). Chloroplast specific DNA region was analyzed and

Table 1. Values of measured traits of investigated forms of alfalfa.

Form of alfalfa	Fertility [%]	Yield of seeds [g plant ⁻¹]	Pod set [%]	Yield of green mass [kg m ⁻²]	Dry mass content [%]
Syn-5	72.1	11.4	73.6	6.38	24.9
Syn-7	35.3	6.3	47.7	3.71	23.4
D ₃	30.8	5.1	34.2	3.85	21.9
D ₅	34.7	3.6	45.2	3.38	20.4
G _{1/1}	39.8	4.2	35.2	3.50	24.2
E _{1/2}	46.3	3.5	30.8	3.12	22.9
E16	34.3	3.3	46.3	2.91	20.1
E51	64.6	6.4	65.8	4.93	20.1
E182	88.5	19.0	88.2	6.36	25.7
E231	89.7	18.2	91.2	6.91	25.6

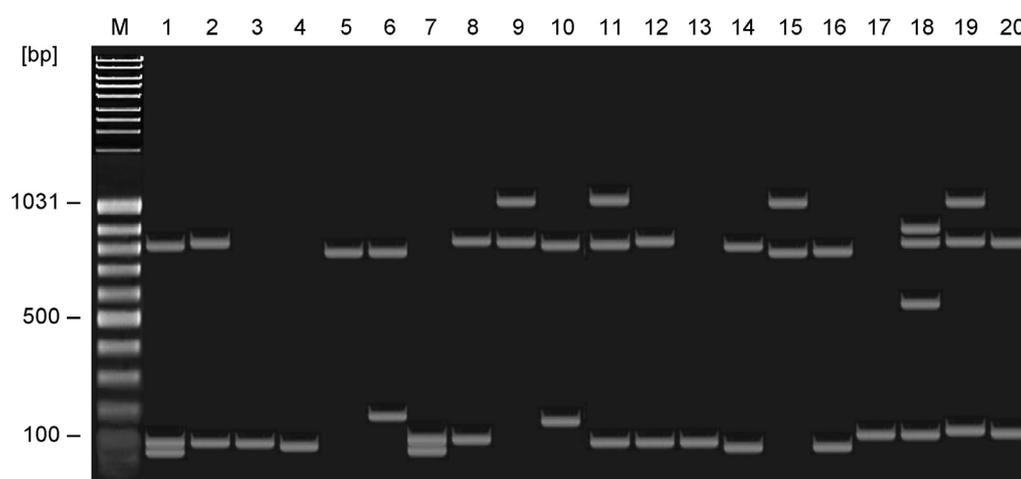


Fig. 1. PCR amplification products with primers SF3 with SR1041 (*lines 1 - 10*) and SF6806 with SR1041 (*lines 11 - 20*); M - marker DNA (MassRuler™ DNA ladder); *lines 1 and 11 - D₃*; *lines 2 and 12 - D₅*; *lines 3 and 13 - G_{1/1}*; *lines 4 and 14 - Syn-5*; *lines 5 and 15 - Syn-7*; *lines 6 and 16 - E_{1/2}*; *lines 7 and 17 - E16*; *lines 8 and 18 - E51*; *lines 9 and 19 - E182*; *lines 10 and 20 - E231*.

Table 2. Dice similarity matrix of 10 forms of alfalfa.

	D ₃	D ₅	G _{1/1}	Syn-5	Syn-7	E _{1/2}	E16	E51	E182	E231
D ₃	100.0	61.0	43.9	57.7	58.2	48.7	55.6	36.7	24.7	32.9
D ₅	61.0	100.0	78.1	96.1	32.1	74.0	36.3	28.3	0	49.0
G _{1/1}	43.9	78.1	100.0	81.9	0	60.8	41.9	31.8	0	56.8
Syn-5	57.7	96.1	81.9	100.0	26.2	76.4	37.4	29.0	0	50.4
Syn-7	58.2	32.1	0	26.2	100.0	20.1	0	21.2	34.1	0
E _{1/2}	48.7	74.0	60.8	76.4	20.1	100.0	30.9	24.8	0	41.6
E16	55.6	36.3	41.9	37.4	0	30.9	100.0	48.5	35.3	29.6
E51	36.7	28.3	31.8	29.0	21.2	24.8	48.5	100.0	67.0	45.4
E182	24.7	0	0	0	34.1	0	35.3	67.0	100.0	25.3
E231	32.9	49.0	56.8	50.4	0	41.6	29.6	45.4	25.3	100.0

mtDNA sequences developed from soybean. The authors found that mtDNA separated entries better than cpDNA markers. In our study, mitochondrial genes differentiated the investigated forms of alfalfa.

The genetic similarity (Dice coefficient) between studied forms of alfalfa (*Medicago sativa* L.) ranged from 20.1 to 96.1 (Table 2). The greatest similarity was noticed between D₅ inbred line and the population Syn-5. The landraces E51 and E182 with the highest seed yield per

plant were distant to the Syn-5. The genetic similarity between these landraces and inbred lines was very low but between the inbred lines it was high. Dendrogram (UPGMA) (Fig. 2) presents the distribution of ten forms of alfalfa into two groups according to the genetic similarity. The first group includes the landraces E16, E51 and E182, the second the remaining forms of alfalfa. The second group is additionally split into three subgroups as follows: the first subgroup included Syn-7

and inbred line D₃, the second lines G_{1/1}, E_{1/2} and E231; the third group consisted of Syn-7 and line D₃.

The three high yielding landraces were clearly separated from the rest of entries. Similarly, the highest resemblance was between Syn-5 and line D₅ and between Syn-7 and line D₃. Taking into consideration the composition of Syn-5 and Syn-7, it seems that the

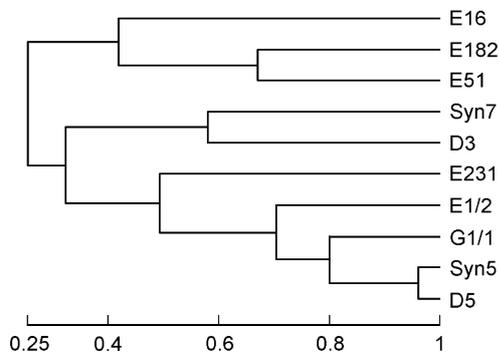


Fig. 2. Dendrogram presenting genetic similarity of ten forms of alfalfa based on the Dice coefficient and constructed by UPGMA.

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substitution of D₃ by E_{1/2} in Syn-7 was not good. Probably this line has no favourable alleles that complement different alleles in the rest of the lines. If the landraces which are characterized by the very high seed yield and yield of green mass contain different alleles complemented alleles of inbred lines, then combination and enrichment of these alleles in a single population could improve the combining ability with synthetic populations of alfalfa. The obtained results showed that the cytoplasms of the investigated alfalfa forms are differentiated. In the breeding programs of alfalfa, the selection of valuable plants follows mother line. Cytoplasmic traits are inherited after mother plants (Forsthoefel *et al.* 1992). This suggests that the characterization of populations of alfalfa based on mtDNA may be very fruitful for breeding program.

In conclusion, the high level of genetic variation between the studied populations of alfalfa indicates the usefulness of these mitochondrial genes for such types of investigations. These populations can be treated as good genetic sources for the selection of the best genotypes for the development of excellent synthetic cultivars of alfalfa.