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EVALUATION OF THE GENETIC VARIABILITY CORRELATED WITH MULTILEAFLET TRAIT IN ALFALFA

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Abstract

The multileaflet trait on alfalfa determine high biomass and increased protein content, therefore the understanding of the metabolic pathway controlling this trait it is of great importance. Different mechanisms were already described, but none of them give a complete explanation for this phenotype. The aim of our work was to evaluate the genetic variability for 8 different plants, both tri and multileaflet ones, originated from MF 42-96 alfalfa synthetic line developed by ICDA Fundulea and wild-type cultivated alfalfa, based on ISSR (Inter Simple Sequence Repeat) markers. Eight ISSR markers were selected from a collection of 20 primers, due to their high polymorphism among the analyzed samples. All the primers generated 157 fragments of which 122 (77.7%) were polymorphic. The evaluation with the UPGMA (unweighted pair-group method) clustering method generated information regarding variability and the dendrogram. The determined similarities coefficients range between 0.44 and 0.64, pointing out a medium variability between samples, with no correlation between phenotypes. In the same clusters were present both tri and multileaflet plants. However, seven polymorphic fragments were identified, present only in multileaflet individuals fingerprints, but absent for all the trileaflet ones. These fragments could be possible candidates to develop a microsatelite markers linked with the multileaflet phenotype on alfalfa.

Key words*: alfalfa, multileaflet, ISSR markers.*

INTRODUCTION

Alfalfa is an important forage plant, with a wide distribution throughout the world. It is cultivated on large areas in Romania due to its high yield and the increased content of crude protein. Besides it has a great importance in crop rotation because it improves the soil with important quantities of nitrogen fixed by symbiotic processes.

This specie is subject to continuous breeding processes aimed to forage yield, forage quality improvment, disease resistance, drought resistance, winter hardiness or creating varieties with high potential in seed production. In germplasm used for alfalfa breeding there are a number of traits which determined the feed quality. Of these, a great importance has the multileaflet trait, identified long time ago in a population of the American Multiking variety (Bingham and Murphy, 1965). The studies regarding the inheritance of this trait pointed out the appearance with a high frequency of the multileaflet leaves (5-10 leaflets) in descendanc (Sheaffer et al., 1995).

Therefore the deciphering of the metabolic pathway which induce the multileaflet trait it is of great importance due to the high biomass and the increased protein content. However this process is very difficult because alfalfa is an allogamus specie, with a tetraploid genome, with a complex process of leaves morphogenesis.

Based on the literature data it was supposed that the multileaflet trait in *Medicago sativa* is the result of different expression of the *knox* genes encoding homeodomain-containing transcription factors involved in meristem development and leaf morphogenesis (di Giacomo et al., 2008; Popescu et al., 2010; Popescu et al., 2014).

The most recent researches suggest that the multileaflet trait is conditioned by the overexpression of some genes primarily involved in maintaining leaf meristems in a state of undetermination for longer time through the control of the genes encoding the plant hormones with morphogenesis functions (Bar and Ori, 2014).

The metabolic pathway is not known yet, the genes overexpression being also controlled by some internal factors (microRNAs) that are likely directed by others hereditary units.
Nevertheless, for an alfalfa genotype without any history of multileaflet trait in ascendancy, there is a very low possibility for the appearance of this trait, heredity being a very important factor.
In order to support these findings there are the observations emphasizing that through repeated self-pollination processes of a multileaflet individual, the percent of multileaflet descendants is increasing from one generation to the other, reaching 70% over four generations. Thus many multileaflet cultivars were obtained based on classical breeding procedures (Odorizzi et al., 2015).
Our research starts-up from the MF 42-96 alfalfa synthetic line, developed by ICDA Fundulea which is characterized by the appearance of multileaflet trait, in a percentage that has not been established because of its lack of stability.
The aim of this work was to develop a process of phenotypic and molecular screenings of alfalfa synthetic line that manifest the multileaflet trait for further development of microsatellite markers linked with this trait.
For the molecular evaluation the ISSR (Inter Simple Sequence Repeat) markers were used. They are based on a repeat sequence from the genome, amplifying the regions located between microsatellite sequences. The ISSR markers were succesfuly used for variability evaluation for a for a wide range of species (Pradeep Reddy et al., 2002).

MATERIALS AND METHODS

As biological materials five individuals originated from MF 42-96 alfalfa synthetic line developed by ICDA Fundulea with different leaf shapes were analyzed. Three genotypes were multileaflet, namely F-MF1, F-MF2, and F-MF3, with different percents of multileaflet leaves and different shapes. Two genotypes were three leaflets, F-TF1 and F-TF2, originated from the same line MF 42-96. Besides, two wild genotypes with multileaflet leaves (W-MF1 and W-MF2) were identified in spontaneous flora and also a wild trileaflet plant as a control (W-TF1) (Table 1).

Table 1 The biological materials used for molecular screening

Code	Origin	Multi-leaflet trait	Leaf shape	Number of leaflets
F-MF1	MF-42-96	Yes 70%	Compound palmate and odd pinnate	5
F-MF2		Yes 60%	odd pinnate	5
F-MF3		Yes 60%	Compound palmate	5
F-TF1		no	odd pinnate	3
F-TF2		no	odd pinnate	3
W-MF1	Wild	yes		5, 7, 9
W-MF2		yes		5
W-TF1		no		3

Biological material production
One multileaflet individual originated from MF 42-96 alfalfa syntetic line was self-polinated. From the resulted seeds, 30 were germinated and then planted in soil. The plants were observed during the next four weeks and the already mention phenotypes were identified.
The wild plants were transferred from field to growth chamber and maintained in the same conditions of temperature (24º C) and lighting (cycle 8/16 hours).

DNA extraction
50 mg of fresh leave tissue from each sample were collected and used for DNA extraction based on CTAB modified method (Doyle and Doyle, 1987).
After isolation and purification the DNA concentration was determined with UV-VIS Nanodrop 8000 spectrophotometer and the samples were diluted to 50 ng/µl concentrations.

DNA amplification
Based on our previous research, 8 ISSR markers were selected due to their high number of amplified fragments: A_2 - $(ACTG)_5$, A_3- $(GACA)_5$, A_{12}- $(GA)_6CC$, A_{13}- $(GT)_6CC$, A_{17}- $(GTG)_3GC$, A_{21}- $(CA)_6AC$, UBC810 - $(GA)_8T$ and UBC824- $(TC)_8G$.
All the DNA samples were amplified successively with the selected primers. For DNA amplification KAPA2G Fast ReadyMix PCR Kit was used (KAPA2G Fast DNA Polymerase 0.5 U/25 µl reaction in a buffer containing 0.2 mM of each dNTP and 1.5 mM $MgCl_2$ at 1X).

The amplication mixture was as follows: KAPA2G Fast ReadyMix (2X) - 12.5 µl, sterile distillate water 9.5 µl, primer (20 µM) - 1 µl, supplementary $MgCl_2$ (25 mM) - 1 µl and DNA (50 ng/µl) - 2µl.
The amplification program was specific for the PCR kit: denaturation - 94°C, 3 min, 45 cycles: denaturation 94°C, 30 sec, primer annealing 52-54°C, 45 sec and DNA synthesis 72°C, 2 min and final extension 72°C, 5 min.
The amplification products were analysed by 1.5% agarose gel electrophoresis, compared with the molecular weight marker PCR Sizer 100 bp (Norgen, Canada). The amplified fragments were evaluated with VisionWorks® LS, (UVP, Anglia).
The software automatically identify and evaluate the size for each band and the data are displayed in a tabular form. The bands are arranged according to the amplicons sizes, being possible to identify polymorphisms between individuals on the basis of the molecular fingerprint obtained through amplification with a specific primer (Guo and Elston, 1999).
The primary data were statistically evaluated based on UPGMA (unweighted pair-group method) clustering method.

RESULTS AND DISCUSSIONS

During four weeks the morphological observations allowed the identification of the five distict phenotypes for the line MF 42-96 descendants. The leaves samples were collected both from those five phenotypes and from the three spontaneous plants - two with multileaflets and one wild, with trileaflets leaves. The DNA was extracted from all of the samples, diluted to 50 ng/µl and analyzed in series with each of the eight selected ISSR markers.
The amplified fragments were separated by agarose gel electrophoresis and their fingerprints and sizes were determined with the specific software. The pictures were shown only for the primers generating the highest (A_{13}) and the the lowest number of fragments (UBC 824) respectively.
For the primer A_2, with $^{5'}(ACTG)_5{}^{3'}$ sequence a number of 17 amplicons were identified, with molecular weights between 320 bp and 1000 bp. All of the alleles were polimorphic, the most obvious diferences being observed in the area of medium molecular weight (360-765 bp).
For the primer A_3, with $^{5'}(GACA)_5{}^{3'}$ sequence, 17 alleles were also identified, with molecular weights between 150 bp and 880 bp. The analysed individuals have four common alleles (215 bp, 400 bp, 450 bp and 630 bp), the rest of 13 being polymorphic; the most obvious differences were in the area of medium molecular weight amplicons (740-880 bp).
A number of 30 alleles were identified with the primer A_{12}, $^{5'}(GA)_6CC^{3'}$ sequence, with molecular weights between 200 bp and 6000 bp. Five alleles were common for all of the individuals (200 bp, 450 bp, 640 bp, 690 bp and 945 bp), the rest of 25 being polymorphic, with differences for all medium, high and very high amplicon sizes (730 - 6000 bp).
The primer A_{13}, with sequence $^{5'}(GT)_6CC^{3'}$ generated 31 alleles, the molecular weights being from 380 bp to 6850 bp. The data analysis pointed out 8 common alleles (515 bp, 695 bp, 765 bp, 830 bp, 1300 bp,1600 bp, 4250 bp and 6000 bp), the other 23 being polimorphic, with differences in the areas of medium and high sizes (870- 3200 bp) (Figure 1).

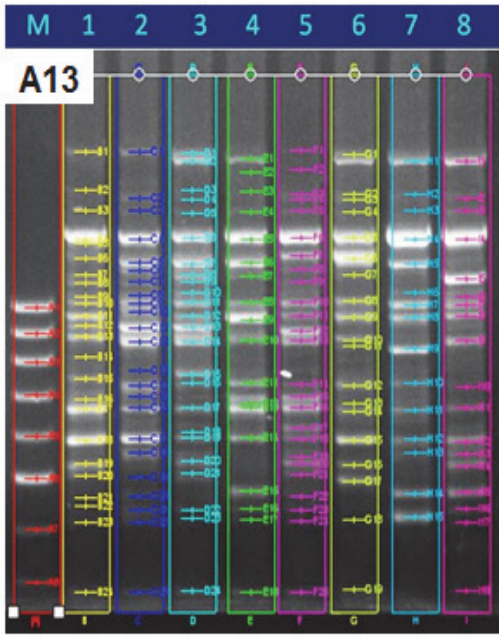

Figure 1. The electrophoretic fingerprint obtained with the ISSR marker A_{13}: (1)- F-MF1, (2) F-MF2. (3)- F-MF3, (4) F-TF1. (5)- F-TF2, (6) W-MF1, (7) W-MF2, (6) W-TF1, M- molecular weight marker PCR Sizer 100 bp (Norgen, Canada)

For the primer A_{17} with the sequence $(GTG)_3GC$, a number of 22 alleles were identified, with molecular weights between 470 bp and 4800 bp. The analysed individuals have eight common alleles (470 bp, 550 bp, 660 bp, 690 bp, 730 bp, 770 bp, 830 bp and 1000 bp) the rest of 14 being polymorphic with differences for medium and high sizes (830-4800 bp).

The primer A_{21}, with sequence $^{5'}(CA)_6AC^{3'}$ generated 17 alleles, the molecular weights being from 400 bp to 4500 bp. The data analysis pointed out 7 common alleles (400 bp, 515 bp, 590 bp, 625 bp,750 bp, 800 bp and 1250 bp), the other 10 being polimorphic, with differences in the areas of medium (850 -1000 bp) and high sizes (3000-4500 bp).

A number of 14 alleles were identified with the primer UBC810, $^{5'}(GA)_6T^{3'}$ sequence, with molecular weights between 400 bp and 4500 bp. Three alleles were common for all of the individuals (500 bp, 5500 bp and 1600 bp), the rest of 11 being polymorphic, with differences for medium amplicon sizes (590-850 bp).

The primer UBC 824, with sequence $^{5'}(TC)_6G^{3'}$ generated 9 alleles with molecular weights between 380 bp and 950 bp, all of them polymorphic, with differences for all the amplicons (Figure 2).

All the primers generated 157 fragments of which 122 (77.7%) were polymorphic, with with an average of 19.6 alleles/primer.

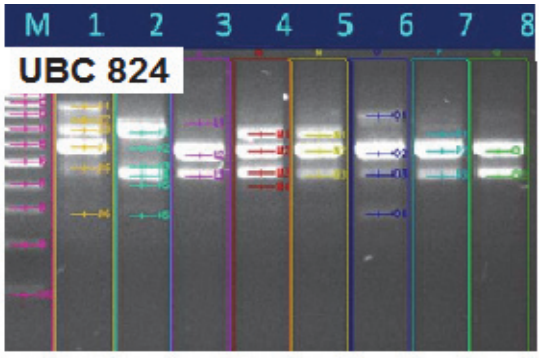

Figure 2. The electrophoretic fingerprint obtained with the ISSR marker UBC 824: (1)- F-MF1, (2) F-MF2. (3)- F-MF3, (4) F-TF1. (5)- F-TF2, (6) W-MF1, (7) W-MF2, (6) W-TF1, M- molecular weight marker PCR Sizer 100 bp (Norgen, Canada)

Taking in account that ISSR are dominant molecular markers the results were evaluated as follows: the presence of the band/allele for one individual was marked as 1 and the absence with 0. Finally, for each primer a binary matrix was established (1, 0); all of the results were statistically analyzed, being possible to establish the similarity coefficients and the dendrogram between the investigated samples.

First, the polymorphism rate was evaluated for each primer (Nagy et al., 2012).

As it was expected for a dominant marker such as ISSR, the PIC (Polymorphism Information Content) values were between 0 and 0.5 (Table 2).

Table 2 Polymorphism rate for the alfalfa genotypes using ISSR primers

No	Primer	Sequence	Bands number		Polimorphism (%)	Bands size (bp)	PIC $\bar{x} \pm s_{\bar{x}}$	PI
			Total	Polimorphic				
1	A2	(ACTG)5	17	17	100	320-1000	0.381±0.028	6.469
2	A3	(GACA)5	17	13	76.47	150-880	0.370±0.027	4.813
3	A17	(GTG)3GC	22	14	63.63	470-4800	0.431±0.021	6.031
4	UBC 810	(GA)8T	14	11	78.57	270-1600	0.366±0.037	4.031
5	A12	(GA)6CC	30	25	83.33	200-6000	0.404±0.019	10.094
6	A13	(GT)6CC	31	23	74.19	380-6850	0.398±0.015	9.563
7	A21	(CA)6AC	17	10	58.82	400-4500	0.290±0.046	3.188
8	UBC 824	(TC)8G	9	9	100	380-950	0.326±0.036	2.938

Primer A17 had the highest contribution to the polymorphism, with a PIC value of 0.431±0.021. The smalest contribution was determined for the primer A_{21} (0.290±0.046).

Based on the values of discrimination index (PI) it was emphasized that markers A_{12} and A_{13} had the highest capacity to generate polymorphic amplicons for the analyzed sample (10,094 and 9,563 respectively). There are no correlations between the primer sequence (2, 3 or 4 repetitive nucleotides) and the values of discrimination index but it was pointed out that ISSR markers are suitable for variability evaluatin on alflafa.

Following UPGMA clustering method the similarities coefficients were determined ranging from 44% to 64% and the dendrogram was established (Figure 3).

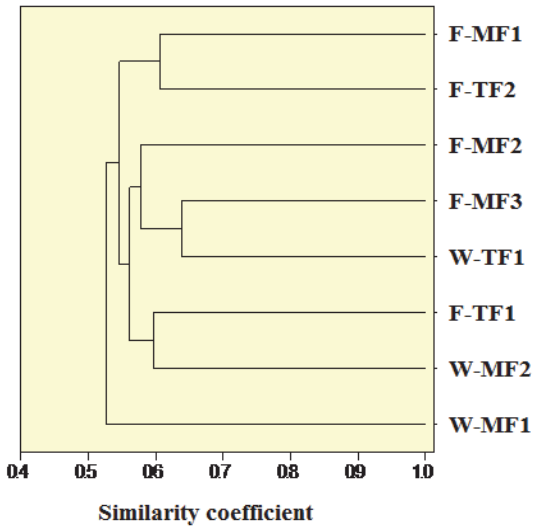

Figure 3. UPGMA clustering of alfalfa genotypes using ISSR primers

Statistical analysis emphasized that the samples were classified in two clusters with a genetic similarity of 44.62%. The individuals from the first cluster are grouped in three subclusters as follows: F-MF1 with F-TF2, with a similarity of 55.74%, F-MF3 with W-TF1, with a similarity of 63.79%, connected with F-MF2 (58.47% similarity) and F-TF1 with W-MF2 with a similarity of 59.63%. In the second cluster a single individual was present, namely W-FM1.

The average similarity for all of the experimental variants was 55.4±3.9%. This value underlines a medium variability, with very close similarities between all the samples. Therefore there is no correlation between the same phenotypes, taking in account that the highest similarity was observed between a multileaflet plant (F-MF3) and a wild trileaflet one (W-TF1).

Considering that the results of statistical analysis do not emphasised differences between different phenotypes, the fingerprints generated by all the eight primers were supplementary analysed. The fragments presents only on multileaflets plants are listed below.

For the primers A_2 a posible candidate was observed, namely the 360 bp amplicon, which was present to the individuals F-MF1, F-MF2 and W-MF1 and W-MF2.

For primer A_3 it was not identified any fragment specific for the multileaflet phenotype.

Using the primer A_{12} a fragment with the length of 2050 bp was identified to F-MF1, F-MF2, F-MF3 and W-MF1 and one of 1020 bp was present to F-MF2, F-MF3 and W-MF1.

For the primer A_{13} the 605 bp fragment was identified as possible candidate, because it was present for F-MF1, F-MF2, F-MF3 and W-MF1.

For A_{17} primer no specific fragments for the multileaflet phenotype were observed.

Using the primer A_{21} a fragment with 3000 bp length was identified for F-MF2 and for the individuals F-MF2 and W-MF1 the 700 bp fragment was specific.

For the primer UBC 810, the allele of 650 bp was present only on the multileaflet individuals originated from the MF 42-96 line (F-MF1, F-MF2, F-MF3)

For UBC 824 it was not identified any fragment specific for the multileaflet phenotype.

Therefore, for the primers A_3, A_{17} and UBC 824 it was not possible to identify amplicons that may be linked with the multileaflet trait. All the amplified fragments were present both for tri and multileaflets individuals. With the other primers seven amplicons were identified, present only in multileaflet phenotypes (Table 3).

Table 3. The presence of the specific alleles in different plant samples

No.	Alele/primer	F-MF1	F-MF2	F-MF2	W-MF1	W-MF1
1	360/A_2	x	x		x	x
2	2050/A_{12}	x	x		x	
3	1020/A_{12}		x	x	x	
4	605/A_{13}	x	x	x	x	
5	3000/A_{21}		x			
6	700/A_{21}		x		x	
7	650/UBC 810	x	x	x		

The amplicon 605/A_{13} was present in four individuals from five and 650/UBC 810 was observed only in plants originated from 42-96 line.

These fragments were specific for the multileaflet plants, pointing out a possible correlation with the specific phenotype.

CONCLUSIONS

Morphological and molecular polymorphism among the descendants of alfalfa synthetic line 42-96 MF was high, emphasizing five distinct phenotypes, both tri and multileaflet, with very different fingerprints.

The ISSR markers were successfully used to evaluate the DNA variability, with high values for the amplicon number - average 19.6 alleles/ primer.

The statistical analysis pointed out a medium average similarity between all of the analyzed samples, therefore it was not possible to establish correlations between different phenotypes.

However, from the fingerprint of each primer the fragments present only on multileaflet plants were determined. The seven amplicons identified only in multileaflet plants, are possible candidates for further investigations.

The researches will be extended to develop a microsatelite markers linked with the multileaflet phenotype on alfalfa.

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