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Intra-Population Genetic Diversity in Romanian Alfalfa Cultivars as Revealed by SSR Markers

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Abstract

The success of a breeding program depends on the genetic variability available into the germoplasm of the crop. The aim of the present study was to evaluate intra-population genetic diversity of the 30 alfalfa Romanian cultivars using three available codominant microsatellite markers (SSRs) identified in the model legume Medicago truncatula from EST database(AFca11, AFct32, AFct45). Genetic diversity was analysed by an autotetraploid statistical method. It was calculated the effective number of alleles, the allele frequencies and estimated heterozygosity. No attempts have been made so far to characterize Romanian alfalfa populations using SSR markers. Our results revealed that the efective number of alleles ranged between 2.00 and 2,92, at the AFca11 locus, between 2.00 and 3.00, at the AFct32 locus, and between 2,00 and 3,78, at the AFct45 locus. AFct32 primer can be considered the most effective for the analysis of genetic diversity in Romanian populations, as revealed heterozygosity amplitude of 49.30%, diversity among genotypes of 48.60% and a reduced probability (0.379) that two individuals chosen at random from the population have the same genotype.

Keywords: intra-population genetic diversity, alfalfa, SSR markers

Introduction

Alfalfa (*Medicago sativa* L.) is the most cultivated forage legume, with about 32 millions hectares over the world (Michaud R. et. al., [1]). Cultivated alfalfa is an allogamous tetraploid with polysomic inheritance, and individuals can have up to four different alleles at a given locus (Quiros C.F. et. al.; Stanford E.H., [2,3]). However, the genetic progress in this species is slow because of its autotetraploidy (Stanford E.H., [3]), with 2n = 4x = 32, its allogamy and the synthetic structure of the varieties. The higher forage yields associated with increased heterozygosity were initially explained by intralocus allelic interaction (overdominance) (Bingham E.T., [4]). Its agronomical interest is based on its high protein content, suitable feeding value and favourable environmental balance (perenniality and no nitrogen fertilizer required).

The genetic diversity is one of the most important factors for crop improvement. Developments in plant molecular biology and DNA analysis have generated different types of methods and markers over the last 10 to 15 years. A large number of SSRs generated by PCR and primer pairs flanking microsatellite sequences are now becoming available for studies on different plant species (Baquerizo-Audiot E. et. al., [5]). Microsatellites are tandemly repeated short DNA sequences that are favored as molecular-genetic markers due to their high polymorphism index. Plant genomes characterized to date exhibit taxon-specific differences in

frequency, genomic location, and motif structure of microsatellites, indicating that extant microsatellites originated recently and turn over quickly (Mun Jeong-Hwan et. al., [6]).

Ellwood et al. [7] used six simple sequence repeat (SSR) loci to analyze the genetic diversity and relationships between randomly selected specimens from 192 accessions in the core *M. truncatula* collection. It thus seems that SSR markers would be a powerful molecular approach for assessing genetic diversity and germplasm characterization in tetraploid alfalfa. Joulier et al. [8] used 87 SSR primer pairs, most from *M. truncatula* ESTs, for genotyping and mapping tetraploid alfalfa populations and the utilization of SSR markers in alfalfa is reported in several other studies (Diwan N. et. al., [9 10]; Mengoni A. et. al., [11, 12,]; Eujayl I.M. et. al., [13]; Flajoulot S. et al., [14]; Sledge M. et al., [15]). Even so, relatively little is known about the utility of SSR markers in elucidating the genetic relationships within and among populations of cultivated alfalfa because of the autotetraploid inheritance of alfalfa and codominant characteristic of SSR marker (Falahati-Anbaran M. et. al., [16]).

In the present paper we present the results obtained with three SSR markers and a new statistical method (Liu Z.P. et. al., [17]) used to investigate the genetic variability within cultivated autotetraploid alfalfa genotypes. This methodology can be used to gain a better understanding of biodiversity conservation and the utilization of genetic resources for use in designing breeding programs for cultivated alfalfa.

Materials and Methods

Plant material. 30 lines and cultivars were involved in this study: Satelit, F 1109-99, F105- 90, Granat, Cosmina, Sigma, F1615-04, F1206-00, F1306-01, F1822-06, Super, F270-91, F1413-02, F1310-01, F1111-99, Pastoral, Magnat, Alina, Selena, Stolo-13, Mf 42-96, Viking, Cristal, F219-91, Coral, Dorina, Saturn, Opal, Venus, F907-97. The seed material was obtained from department working in alfalfa breeding from I.N.C.D.A. Fundulea.
Ten *in vitro* growing plants per population were randomly selected, and green healthy leaves from each plant were collected for DNA extraction.

SSR analysis

DNA extraction. Total genomic DNA from 300 plants was extracted using Maxwell™ 16 Instrument from Promega. Purified concentrated products are obtained at high quality and high yield and can be used directly in a variety of downstream applications.

Primers and SSR-PCR assay. The number of 16 SSR primer pairs was screened using ten DNA samples from each genotype and on the basis of this preliminary data, 3 polymorphic SSR primers (AFca11, AFct32, AFct45) were chosen. In table 1 are presented the sequences of the 3 primers used for detection of the genetic polymorphism within alfalfa cultivars (Falahati-Anbaran M. et. al., [16]).

Table 1. The three pairs of alfalfa polymorphic simple sequence repeat (SSR) primers used in this study

Loci	Forward (F) and reverse (R) primers	Allele size (pb)
AFca11	(F) CTTGAGGGAACTATTGTTGAGT (R)AACGTTTCCCAAAACATACTT	150-300
AFct32	(F) TTTTTGTCCCACCTCATTAG (R) TTGGTTAGATTCAAAGGGTTAC	80-250
AFct 45	(F)TAAAAAACGGAAAGAGTTGGTTAG (R) GCCATCTTTTCTTTTGCTTC	130-300

The amplifications were carried out in a 25 µl PCR buffer containing: GoTaq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, $MgCl_2$, and reactions buffers at optimal concentrations for efficient amplification of DNA templates), SSR primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during eletrophoresis. DNA amplification was carried out using a Thermalcycler by Corbett and reactions were submitted to the following PCR program: preliminary DNA denaturation for 1,3 min at 94°C, followed by 32 cycles consisting of denaturation (30 min, 94°C), primer annealing (1 min, 65-55°C), and extension (1 min, 72°C). A final extension for 4 min at 72°C was included. The SSR products were separated by 2% agarose gels electrophoresis (3V cm-1), which run with 1xTAE buffer. The PCR marker (1000-50bp) was also run on each gel as a molecular weight standard. Photo documentation was performed under UV light using a photo imaging system.

Data analysis

The heterozygosity (*h'*) was calculated using the same formula as for the binary heterozygosity (*h*) (Anderson J.A. et. al., [18]).

$$He = 1 - \sum_{i=1}^{n} P_i^2 \; ; \qquad Pi = \frac{1}{N} \sum_{i=1}^{n} fi \; ;$$

Where: - *fi* is the presence or absence of a specific band in each plant; *n* is the number of plants in the population; *N* is the total number of locus *i* bands in a population.

The allele frequencies (P_i) and the effective number of allele (*Ne'*) were also calculated (Liu Z.P. et. al., [17]).

$$\overline{P}_i = \frac{P_i^{'}}{P_E + P_0} \; ;$$

$$P_i^{'} = \frac{5}{2} \sum_{i=1}^{n} \alpha_i + \frac{5}{3} \sum_{i=1}^{n} \beta_i + \frac{5}{4} \sum_{i=1}^{n} \chi_i + \sum_{i=1}^{n} \varepsilon_i \; ;$$

$$P_E = \frac{3}{2} \sum_{i=1}^{n} \alpha_i + \frac{2}{3} \sum_{i=1}^{n} \beta_i + \frac{1}{4} \sum_{i=1}^{n} \chi_i \; ;$$

$$Ne = \sum_{j=1}^{n} \frac{1}{n.(\sum_{i=1}^{j} \overline{P}_i^4 + 4P_0 \sum_{i=1}^{j} \overline{P}_i^3 + 6P_0^2 \sum_{i=1}^{j} \overline{P}_i^2 + 4P_0^3 \sum_{i=1}^{j} \overline{P}_i + P_0^4)} \; ;$$

Where: - *Pi'* is the expected number of the j allele (expect for the null allele) of a locus; P_E is the sum of *Nj* in locus *i*; P_0 is the expected number of null alleles for a locus.

The banding phenotypes are given by α= one band, β= two bands, χ= three bands and ε= four bands.

To estimate the intra-population genetic variability was used Shannon-Weawer diversity index (Ciulca S., [19]).

$$J = \frac{H^{'}}{H_{\max}} ; \quad H^{'} = -\sum_{i=1}^{s} p_i \log p_i \; ; \quad H_{\max} = \log s \; ; p_i = f_i/n$$

Were: *s*- number of observations categories; p_i- frequency of observations from *i* category; *n*- total number of sample observations; f_i – number of observations from *i* category.

Results and Discussions

Number of bands generated by different pairs of primers used was scored. The frequencies of the SSR fragments were estimated for each of individuals from each genotype. In figure 1 is showed an example of SSR banding patterns variation at the Selena and Stolo13 individuals for AFct 32 locus.

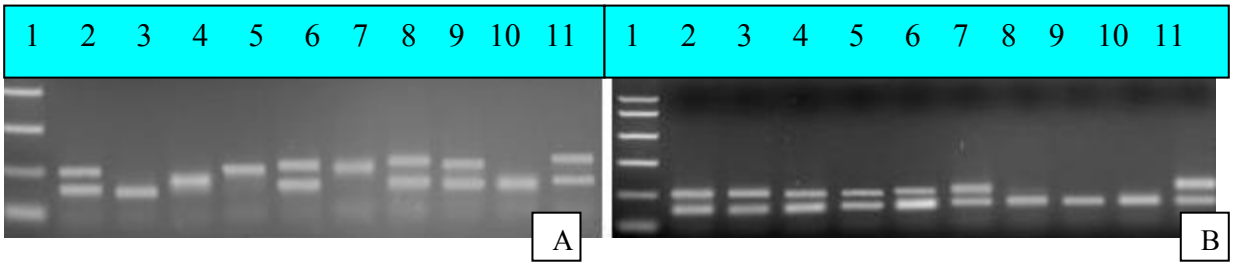

Figure 1. Analysis of 2% agarose gel electrophoresis for amplified products using AFct 32 primer (A)- Selena, (B)- Stolo 13 genotypes ; 1 - molecular marker; 2-11 individuals.

The results of the genetic diversity evaluation at the level of analyzed loci showed the highest frequency of heterozygosity (*He*) at F907-97 (52.70%), F1822-06 (51.30%), Cosmina (51.30%), Satelit (46.70%) and Dorina (43.30%) lines, respectively, cultivars. The higher frequency of homozygosity was observed in populations derived from F1615-04 (83.30%), Sigma (82.00%), Alina (75.30%), Stolo 13 (74.70 %) and F1206-00 (74.70%).

The average number of alleles (*Ne*) estimated for the three loci ranged from 2.22, at Sigma variety, and 3.16, at line F 907-97. There was a direct correlation between estimated and effective number of heterozygous alleles, genotypes possessing multiple alleles at a locus are also remarkable through a high frequency of heterozygosity (Table 2).

Table 2. Estimated heterozygoty, effective alleles number and diversity index in studied genotypes using SSR primers

No crt	Genotype	*He* $\bar{x} \pm s_{\bar{x}}$	*Ne* $\bar{x} \pm s_{\bar{x}}$	*J*	No crt	Genotype	*He* $\bar{x} \pm s_{\bar{x}}$	*Ne* $\bar{x} \pm s_{\bar{x}}$	*J*
1	SATELIT	0,467±0,077	2,95±0,26	0,729	1	PASTORAL	0,373±0,053	2,62±0,15	0,566
2	F 1109-99	0,407±0,047	2,71±0,13	0,619	2	MAGNAT	0,260±0,080	2,39±0,17	0,503
3	F105-90	0,440±0,050	2,81±0,15	0,638	3	ALINA	0,247±0,127	2,40±0,21	0,479
4	GRANAT	0,407±0,047	2,71±0,13	0,635	4	SELENA	0,333±0,093	2,56±0,23	0,567
5	COSMINA	0,513±0,066	3,14±0,32	0,676	5	STOLO-13	0,253±0,129	2,41±0,22	0,558
6	SIGMA	0,180±0,007	2,22±0,01	0,461	6	MF 42-96	0,413±0,052	2,73±0,15	0,609
7	F1615-04	0,167±0,093	2,23±0,14	0,444	7	VIKING	0,273±0,146	2,49±0,29	0,520
8	F1206-00	0,253±0,129	2,41±0,22	0,511	8	CRISTAL	0,307±0,070	2,47±0,15	0,607
9	F1306-01	0,267±0,141	2,46±0,27	0,530	9	F219-91	0,300±0,151	2,55±0,28	0,546
10	F1822-06	0,513±0,058	3,12±0,27	0,685	10	CORAL	0,380±0,100	2,69±0,23	0,602
11	SUPER	0,353±0,033	2,56±0,08	0,557	11	DORINA	0,433±0,057	2,80±0,17	0,618
12	F270-91	0,413±0,118	2,83±0,32	0,632	12	SATURN	0,380±0,106	2,71±0,28	0,595
13	F1413-02	0,407±0,047	2,71±0,13	0,588	13	OPAL	0,260±0,080	2,39±0,17	0,560
14	F1310-01	0,373±0,097	2,66±0,22	0,598	14	VENUS	0,300±0,151	2,55±0,28	0,523
15	F1111-99	0,267±0,141	2,46±0,27	0,530	15	F907-97	0,527±0,047	3,16±0,24	0,681

A high level of genetic diversity at all the SSR loci was found in all Romanian alfalfa populations studied (table 2). Mean genetic diversity ranged from 0.729 in the Satelit population to 0.444 in the line F 1615-04.

The genetic similarity varied from 0.479 (cultivar Alina) to 0.444 (line F 1615-04), indicating a low level of genetic similarity among individuals within populations.

To assess the discriminatory power of the three primers and their ability to identify genetic differences between genotypes, to appeal to values estimated heterozygosity, genetic diversity index of Shannon-Weawer and the probability of genetic identity. It is considered to be more effective primer that shows high values of heterozygosity and diversity index, correlated with low levels of probability of genetic identity (Korkovelos A.E. et. al., [20]).

AFct32 primer can be considered the most effective for the analysis of genetic diversity in Romanian populations, as revealed heterozygosity amplitude of 49.30%, diversity among genotypes of 48.60% and a reduced probability (0.379) that two individuals chosen at random from the population have the same genotype. AFca11 has the smallest capacity to identify genetic differences in the populations of studied alfalfa varieties (table 3).

Table 3. Heterozygosity estimated, diversity index and the probability of genetic identity for SSR primers used in the alfalfa studied genotypes

Crt. no.	Primer	*He*	*J*	*PI*
1	AFca 11	0,312	0,477	0,522
2	AFct 32	0,493	0,486	0,379
3	AFct 45	0,405	0,632	0,397

SSR analysis of studied genotypes to prove the existence of genetic variability between these genotypes which can be of interest to alfalfa improvement programs to identify valuable genes of combinations.

As the level expected heterozygosity using SSR primers, we see that there are no significant differences between varieties and lines studied. A possible cause could be due to the fact that these genotypes have very complex origin, consisting of at least 6 genotypes with different origin.

Conclusions

The obtained results suggest that SSR markers are useful for identification of alfalfa populations, for assessing inter- and intra-population genetic diversity, and for estimation of genetic differentiation.

Given the three analyzed loci, is observed that the highest frequency of heterozygosity was estimated at F 907-97 (52,70%), Cosmina (51,30%), F 1822-06 (51,30%), Satelit (46,70%) and Dorina (43,30%) genotypes. The highest frequency of homozygous alleles for those loci was observed in F 1615-04 (83,30%), Sigma (82,00%), Alina (75,30%), Stolo 13 (74,70 %), F 1206-00 (74,70%) genotypes. Following the analysis carried out, is noted that there is a high intrapopulation diversity for alleles of these three loci to Satelit (0,729), F 1822-06 (0,685), F 907-97 (0,681), Cosmina (0,676) genotypes. The high homogeneity to those loci alleles was observed in F 1615-04 (0,444), Sigma (0,461), Alina (0,479) genotypes. The average number of alleles estimated for the three loci, showed values ranging from 2.22 to 3.16 in F 907-97 line and Sigma cultivar. Also, at the studied genotypes was observed that there is a direct correlation between estimated and actual number of heterozygous alleles, such that genotypes that possess more alleles at a locus, record a higher frequency of heterozygosity too.

AFct 32 was the most efficient locus in detecting genetic diversity in alfalfa studied varieties, giving a heterozygosity of 49.30%, diversity among genotypes of 48.60%, correlated with a reduced probability (0.379) that two individuals chosen at random from the population have the same genotype.

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.

References

1. Michaud, R; Lehman, WF; Rumbaugh, MD. World distribution and historical development. Alfalfa and alfalfa improvement – Agronomy Monograph no 29, ASA-CSSA-SSSA, Madison, USA. 1988. pp. 25–91.
2. Quiros C.F., Bauchan G.R., 1988, *The Genus Medicago and the Origin of the Medicago sativa complex,* Alfalfa Improvement, Series Agronomy, 29, 93-121.
3. Stanford, EH. Tetrasomic inheritance in alfalfa. Agron J. 1951;43:222–225.
4. Bingham ET (1980) Maximizing heterozygosity in autopolyploids. In: Lewis WH (ed) Polyploidy: biological relevan Stanford, EH. ce. Plenum, New York, pp 471–491.
5. Baquerizo-Audiot E, Desplanque B, Prosperi J, Santoni S., 2001, *Characterization of microsatellite loci in the diploid legume Medicago truncatula*. Molecular Ecology 1:1–3.
1. 6.Mun Jeong-Hwan, Kim Dong-Jin, Choi Hong-Kyu, Gish John, Debelle Frederic, Mudge J., Denny R., Endre G., Saurat O., Dudez A., Kiss B. Gyorgy, Roe B., Young D. Nevin, Cook R. Douglas, 2006, *Distribution of Microsatellites in the Genome of Medicago truncatula: A Resource of Genetic Markers That Integrate Genetic and Physical Maps*. Genetics 172:2541-1555.
6. Ellwood SR, Souza NKD, Kamphuis LG, Burgess TI, Nair RM and Oliver RP (2006) SSR analysis of the *Medicago truncatula* SARDI core collection reveals substantial diversity and unusual genotype dispersal throughout the Mediterranean basin. Theor Appl Genet 112:977-983.
7. Julier B., Flajoulot P., Barre P., Cardinet G., Sylvain S, 2003, *Construction of two genetic linkage maps in cultivated tetraploid alfalfa (Medicago sativa L.) using microsatellite and AFLP markers.* BMC. Plant Biol., 3: 9-9.
8. Diwan N., Bhagwat A., Bauchan G., Cregan P., 1997, *Simple sequence repeat (SSR) DNA markers in alfalfa and perennial and annual Medicago species.* Genome.40**:**887–895.
9. Diwan N., Bouton J., Kochert G., Cregan P., 2000, *Mapping of simple sequence repeat (SSR) DNA markers in diploid and tetraploid alfalfa*. Theor Appl Genet. 101:165–172.
10. Mengoni A, Gori A and Bazzicalupo (2000a) Use of RAPD and mircosatellite (SSR) variation to assess genetic relationships among populations of tetraploid alfalfa, *Medicago sativa*. Plant Breeding 119:311-317.
11. Mengoni A, Ruggini C, Vendramin G and Bazzicalupo (2000b) Chloroplast microsatellite variation in tetraploid alfalfa. Plant Breeding 119:509-512.
12. Eujayl I.M., Sledge K., Wang L., May G.D., Chekhovskiy K., 2004*, Medicago truncatula EST-SSRs reveal cross-species genetic markers for Medicago spp*. Theor. Appl. Genet. 108: 414–422.
13. Flajoulot S., Ronfort J., Baudouin P., Barre Ph., Huguet T., Huyghe C., Julier B., 2005, *Genetic diversity among alfalfa (Medicago sativa L.) cultivars coming from a breeding program, using SSR markers.* Theor. Appl. Genet. 111:1420-1429.
14. Sledge, M. K., I. M. Ray And G. Jiang, 2005 *An expressed sequence tag SSR map of tetraploid alfalfa (Medicago sativa L.).* Theor. Appl. Genet. Aug 2: 1–13.
15. Falahati-Anbaran M, Habashi AA, Esfahany M, Mohammadi SA, Ghareyazie B, 2007, *Population genetic structure based on SSR markers in alfalfa (Medicago sativa L.) from various regions contiguous to the centres of origin of the species.* Journal of Genetics, vol.86, no1.
16. Liu Z.P., Liu G.S., Yang Q.C., 2007, *A novel statistical method for assessing SSR variation in autotetraploid alfalfa (Medicago sativa L.)* Genetics and Molecular Biology, 30, 2, 385-391.
17. Anderson J.A., Churchill G.A., Autrique J.E., Tanksley S.D., Sorrells M.E.,1992, *Optimizing parental selection for genetic linkage maps*. Genome, 36, 181–186.
18. Ciulca S., 2006, *Metodologii de experimentare în agricultură şi biologie*. Ed Agroprint, Timişoara.
19. Korkovelos A.E., Mavromatis A.G., Huang W.G., Hagidimitriou M., Giakoundis A., Goulas C.K., 2008, *Effectiveness of SSR molecular markers in evaluating the phylogenetic relationships among eight Actinidia species.* Scientia Horticulturae 116: 305–310.