INTER- AND INTRA- POPULATIONAL MOLECULAR DIFFERENCES OF SPONTANEOUS *Medicago sativa* (L) GENOTYPES OF CERNAVODA ECOSYSTEMS

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Abstract. *The paper presents the molecular aspect of DNA to wild alfalfa plants grown in two different ecosystems over 3 years. The chosen ecosystems were in the close vicinity of the CNN Plant where emissions are considered by the public to be dangerous and clean areas at a distance of 33 km generically called red and green respectively. The established locations were the CNN Plant yard and the Valea Cismelei for the red zone, respectively Oltina and Vlahi for the green zone. The plant samples were collected in two campaigns (spring and fall); genomic DNA was analyzed by CTAB modified method (Sambrook, 1989); using VisionWorks® LS software (UVP, England); the primary data were statistically processed by variance analysis and the similarity clusters. There were highlighted a total of 685 alleles (27 primers) for Medicago spp. with an average of 25.37 alleles/primer. The similarity of the inter-populations was high and significant, emphasizing a high analogy between the DNA of individuals of red and green ecosystems. Compared to the inter-population similarity the variability of the molecular profile of the individuals from the same location was high. From this point o view the activity of the Cernavoda Nuclear Power Plant has not affected the DNA structure of alfalfa plants.*

Keywords: molecular profile, *Medicago sativa* (L), green and red ecosystems

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1. Introduction

Tritium (^{3}H) is a radioactive isotope of hydrogen (^{1}H) due to interaction of atmosphere with cosmic radiation. The amount of natural ^{3}H is very low as trace (2,590,000 TBq) [8]. From current amount of ^{3}H 11.34 kg the largest part is generated by nuclear plants activity [12]. 93% of ^{3}H stays in hydrosphere and about 7% is in atmosphere [3]. After Chernobyl (1986) and Fukushima Daiichi (2011) disasters the people are more skeptics and concerned to the nuclear energy even if in the both cases a human error generated them. The Cernavoda Nuclear Power Plant (CNN) is a sensitive subject and generally the people's fears of unknown situations as well as the Plant activity. Although nuclear power plants are modern and produce cheap electricity, they have effects on the environment through cooling water, gaseous, heat and radioactive waste emissions such as tritium from CANDU-type reactors. Only the proper knowledge about its effluents involvement with environment can develop strengthen the full confidence of its advantages.

For this reason our work was oriented to analyze the flora and fauna chromosomes evolution and DNA molecular profile.

We considered alfalfa plants as a "tritium effect detector" because the tiny roots are in contact with the soil air, the pivoting ones can reach the groundwater and the large leaf mass ensures that the atmospheric air enters the plant and with this and ^{3}H. The residence time of ^{3}H is shorter if it is bound to free water (TFWT) and longer if it is organically bound (OBT). The high concentration of tritium into environment is involved with cellular functions and be able to produce injury of DNA. Only in this way can it become dangerous, causing cell dysfunction and even hereditary changes.

In order to have an image regarding the changes produced by ^{3}H at the DNAs level, were investigated perennial alfalfa genotypes from 2 ecosystems from the red zone which is near the Cernavoda Plant and 2 ecosystems from the green zone which is clean i.e. CNN yard and Valea Cismelei respectively Oltina and Vlahi.

The *main objective*: to establish the impact of Cernavoda Nuclear Plant operation upon the DNA of alfalfa perennial genotypes from the red and green ecosystems.

2. Materials and Methods

The monitoring work of the CNN influence upon alfalfa DNA was carried out in 3 successive years (2013, 2014, and 2015) in two distinct areas green and red in 2 season spring and autumn.

2.1.Biological Material

Alfalfa plants were marked in the spring of 2013 so that shoots of the same genotype could be identified in the following years 2014 and 2015. The number of annual samples was dependent on Danube floods, plant growth, grazing or other unavoidable accidents (Table 1).

Table 1. Code of molecular analysis samples collected from spring and autumn of 2013, 2014 and 2015

Red area				Green area			
Nuclear Plant (CNN)		Valea Cismelei (CV)		Oltina (O)		Vlahi (V)	
Spring (**P**)	Autumn (**T**)	Spring (**P**)	Autumn (**T**)	Spring (**P**)	Autumn (**T**)	Spring (**P**)	Autumn (**T**)
CNN_{U1}13**P**	CNN_{U1}13**T**	VC_S13**P**	VC_S13**T**	O13**P**	O13**T**	V13**P**	V13**T**
CNN_{U1}14**P**	CNN_{U1}14**T**	VC_S14**P**	VC_S14**T**	O14**P**	O14**T**	V14**P**	-
$CNN_0$14**P**	-	-	-	-	-	-	-
CNN_{U1}15**P**	CNN_{U1}15**T**	VC_{S1}15**P**	VC_{S1}15**T**	$O_1$15**P**	$O_1$15**T**	-	V15**T**
-	$CNN_0$15**T**	VC_{S2}15**P**	VC_{S2}15**T**	-	$O_2$15**T**	-	-
CNN_{STA}15**P**	CNN_{STA}15**T**	-	VC_{S3}15**T**	-	$O_3$15**T**	-	-
$CNN_{P\text{-}TA}$15**P**	$CNN_{P\text{-}TA}$15**T**	VC_{N1}15**P**	VC_{N1}15**T**	-	-	-	-
-	-	VC_{N2}15**P**	VC_{N2}15**T**	-	-	-	-
-	-	VC_{Cen}15**P**	VC_{CEN}15**T**	-	-	-	-

Legend:
The ecosystem name/collection point/year/season.
Some sections are empty because no plants were found or the location was flooded by the Danube.
Source: Own results.

For safety reasons additional samples were collected and processed and displayed in dendrograms. These are: in 2013 Vlahi-$V_2$13P and Oltina-$O_2$13T; in 2014 CNN Plant-CNN_{STA2}14P; in 2015, Valea Cismelei-VC_{N2}15T, VC_{S2}15T and VC_{S3}15T; Oltina-$O_2$15T and $O_3$15T; Lac Tibrin LT15T and Seimeni S15T.

2.2.The working methods

For the samples preparation and DNA extraction modified CTAB method was used [2]. The extracted nuclear DNA from each plant sample was evaluated using the NanoDrop 8000, to establish the concentration and DNA quality the spectrophotometric method was used [9]. The OD 260/280 ratio ranged from 1.8 to 1.9 pointing out the proper quality of samples. Due to the high concentration of DNA, each sample was diluted to 100 ng/ml concentration.

The molecular profile was performed based on RAPD (*Random Amplification of Polymorphic DNA*) and ISSR (*Inter Simple Sequence Repeat*) markers [9, 7] (Table 2).

Due to their low results some markers were eliminated and new ones were introduced.

Table 2. The sequence of the primers type used for the assessment of molecular variability (2013, 2014 and 2015)

No.	Code	Primer name	Sequence 5' → 3'	2013		2014	2015
				Spring	Autumn	in both seasons	
1	Pr 3	UBC810	$(GA)_8T$	Yes	Yes	Yes	Yes
2	Pr 4	UBC811	$(GA)_8C$	Yes	Yes	Yes	Yes
3	Pr 6	UBC816	$(CA)_8T$	Yes	Yes	-	Yes
4	Pr 7	UBC820	$(GT)_8C$	Yes	Yes	-	Yes
5	Pr 17	UBC841	$(GA)_8YC$	Yes	Yes	-	-
6	Pr 24	UBC853	$(TC)_8RT$	-	Yes	-	-
7	Pr 28	UBC859	$(TG)_8RC$	-	Yes	Yes	-
8	Pr 30	UBC864	$(ATG)_6$	-	Yes	-	Yes
9	Pr 34	UBC884	HBH $(AG)_7$	-	Yes	-	-
10	Pr 36	UBC886	VDV$(CT)_7$	-	Yes	Yes	-
11	-	A2	$(ACTG)_5$	Yes	Yes	Yes	Yes
12	-	A3	$(GACA)_5$	Yes	Yes	Yes	Yes
13	-	A7	$(AG)_{10}T$	Yes	Yes	Yes	Yes
14	-	A 12	$(GA)_6CC$	Yes	Yes	Yes	Yes
15	-	A 21	$(CA)_6AC$	-	-	-	Yes

Source: Own results.

The amplification products were separated by agarose gel electrophoresis and evaluated by VisionWorks®LS (UVP, UK) software. All the amplified fragments, visualized as bands in UV light were determined and aligned according to their size (bp). Taking in account that ISSR are dominant markers, which don't allow the differentiation between homo- and hetero- zygotes, they were scored with 1 and 0 for presence and absence respectively.
The obtained results were statistically operated using the *Jaccard Index*/ Similarity Coefficient (Sc) [6]. According to the used primers and based on the molecular similarity index for each year and to report DNAs kinship the UPGMA Dendrogram was constructed (from Figure 1 to Figure 5).

3. Results and Discussions

3.1.The amount, quality and molecular pattern of alfalfa DNA

The horizontal gels of electrophoresis analytical-grade agarose in the running buffer (1X TAE) and a control DNA dye with appropriately sized DNA standards pointed out the difference among the samples.

The DNAs quality and availability to be analyzed was established by OD 260/280 ratio. In all years they ranged from 1.8 to 1.9 pointing out its high quality. Also the DNA quality is in correlation with the molecular weight of bands. In our case the high molecular weight varies from 1,540 bp to 1,000 bp for UBC 811 (4

primers) & UBC 810 and A2 (3 primers). The middle size fragments varied from 980 bp to 500 bp to 4 primers in equal frequency for UBC and A group. The light molecular fragments vary from 490 bp to 175 bp in case of 3 UBC primers and for A2. In terms of molecular fractions number, DNA of plants grown in Valea Cismelei was like to that of plants in Oltina (37.5% and 39.81% respectively). The pattern complexity of DNA from samples collected in CNN yard pointed out less fragments molecular (23.61%), i.e. it's uniformity was higher.

Table 3. The primers and their products (2013, 2014, 2015)

No.	Primers	Number of loci			No.	Primers	Number of loci		
		2013	2014	2015			2013	2014	2015
1	UBC810	1-6	1-24	1-24	10	UBC886	-	1-27	-
2	UBC811	1-17	1-28	1-27	11	A2	1-20	1-24	1-46
3	UBC816	1-13	-	1-27	12	A3	1-33	1-18	1-48
4	UBC820	1-18	-	1-27	13	A7	1-15	1-21	1-31
5	UBC841	1-25	-	-	14	A12	1-12	1-24	1-44
6	UBC853	-	-	-	15	A 21	-	-	1-28
7	UBC859	-	1-19	-	No. of Alleles		159	185	341
8	UBC864	-	-	1-39	No. of Primers		9	8	10
9	UBC884	-	-	-	Allele average /Pimer		17.7	23.1	34.1

Source: Own results.

The primer's products varied yearly being in a large amount in 2015 and the smallest in 2013 (Table 3). The analysis of one and the same primer by year revealed obvious differences. Thus, the UBC 810 primer has the same values in 2014 and 2015, while UBC 811 revealed the maximum in 2014, followed by 2015 and closely was 2013. For the A2, A7, and A12 primers products were yearly more and more effective.

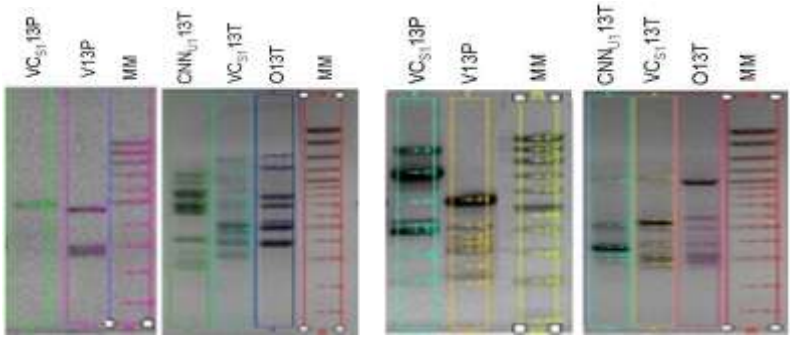
3.2.Season-dependence of DNA profile

At a quickly analysis of the electrophoresis products are seeing the differences between ecotypes but especially between framework of years and season. The autumn DNA profile is different from the spring especially of Valea Cismelei plants. The alfalfa samples displayed for each season a differentiated molecular pattern (Figure 1, Figure 2 and Figure 3).

In the 2013 spring DNAs of VC_S13P and of V13P pointed out only a few product with a small similarity between them. In autumn, for both primers UBC 810 and UBC 811 the molecular configuration were larger and almost identical (Figure 1).

In the 2015 spring between $CNN_{U1}15P$, VC_S15P, VC_N15P, O15P and V15P the molecular profile of DNA were slightly differentiated while in the autumn they

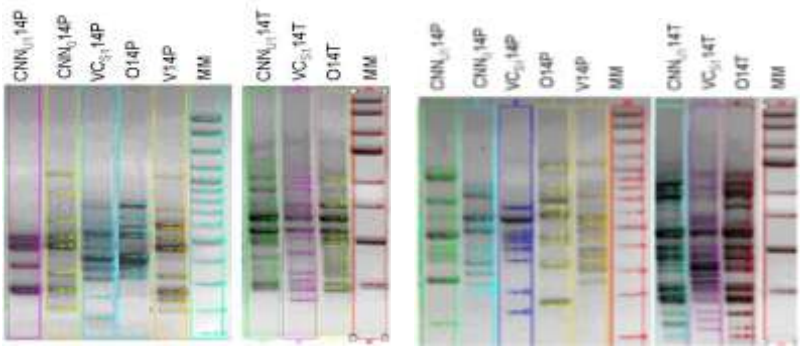
were almost identically (Figure 3). In their profile a lot of large bands were observed.



UBC 810 **UBC 811**

a b

Fig. 1. The spring (P) and autumn (T) DNA profile separately into molecular different weight bands (bp) by UBC810 and UBC811 primers (2013)
Source: Own results.



UBC 810 **UBC 811**

a b

Fig. 2. The spring (P) and autumn (T) DNA separated in bands with different molecular weight (bp) by UBC810 and UBC811 primers (2014)
Source: Own results.

The amplification products was poor in all years and almost to all DNA of spring plants (Table 4).

In comparison to spring DNA the spatial settlement of autumn DNA bands are different being higher in number and width and more complex. Broadbands ranged fromat 1 (CV_{S1}15P) to 3 (CNN_{U1}15P and O15P) in spring genotypes while in the autumn ones there were more ranging from 3 (VC_{S1}15T, VC_{N1}15T and O15T) to 4 (CNN_{U1}15T). The wide bands are in the area of average molecular weight indicating many alleles/locus. In comparison to spring DNA the number of molecular fragments of autumn DNAs was more complex having a higher number

of fragments (59.72%) including 83.33% heavy fragments (1,600 bp-1,000 bp), middle 72.41% (1,000 bp-500 bp) and light 74.07% (490 bp-100 bp).

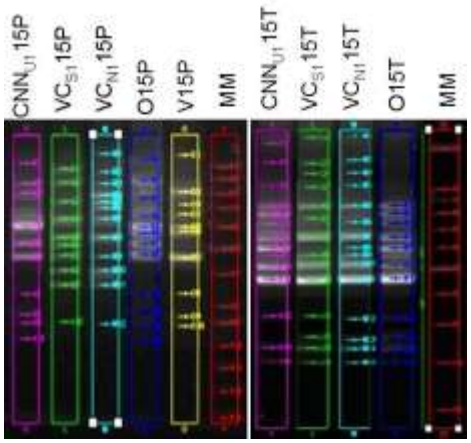


Fig. 3. The UBC 810 primer amplification products of DNA samples of the spring (P) and autumn plants (T; 2015)
Source: Own results.

Table 4. The percent of amplification products bands of different primers grouped by degree of expression of *Medicago sativa* genotypes collected in spring and autumn years 2013, 2014, 2015

Year	Ecosystem	No of primers	% of resulting products that were:		
			Higher in fall than spring (T>P)	*Equal in autumn and spring* (T=P)	Smaller autumn than spring (T<P)
2013	Valea Cişmelei	9	66.67	11.11	22.22
	Vlahi	9	77.77	22.23	0
2014	**Valea Cişmelei**	**8**	**75.00**	**0**	**25.00**
	CNN	**8**	**62.50**	**25.00**	**12.50**
	Oltina	**8**	**87.50**	**0**	**12.50**
2015	Cişmelei Valley	10	50.00	0	50.00
	CNN	10	30.00	40.00	30.00
	Oltina	10	40.00	20.00	40.00
General percent (%)			**59.73**	**15.27**	**25.00**
The areas under the direct influence of the CNN Plant					
Red (VC+CNN)		45	55.56	15.56	28.88
The areas free of the CNN Plant influence					
Green (Oltina+Vlahi)		27	66.67	14.81	18.52

Source: Own results.

The results obtained in the three years highlighted more electrophoretic products in the fall of 2013 and 2014 (Table 4).

In addition to other types of analysis, the electrophoretic products from 2015 were atypical. It should be noted that between the red and green areas the

electrophoretic products were more distinctive for the DNA of autumn than the spring one being higher (55.56% and 66.67% respectively). The equal electrophoretic products for both spring and autumn seasons were of 15% and 14% for red and green areas.

At the plants collected in spring in the red zone, the electrophoretic products were more numerous than those of plants in the green zone (28.88%>18.52%).

3.3.The likenis of ecotypes

Based on the fingerprints the similarity coefficients were established and the dendrograms were designed.

The dendrograms from 2013 and 2014 are quite similar (Figure 4a and 4b). In 2015, due to the large number of samples, dendrograms were made apart for each primer. The choice of dendrograms from 2015 was done by correlation coefficients calculated. Significant correlation was between the group of primers A2-UBC 811-UBC 816 (0.358***, 0.788***>r0.1%), respective UBC 810-A3-A21 (0.606***, 0.545***>r0.1%=0.230). Farther will be discussed only these two dendrograms (Fig. 5a and 5b).

In 2013 the dendrogram contains 2 clades (Figure 4a). The CNN_{U1}13P genotype was willing separately from the two clades. In first clade are close connected VC_S13P respectively O13P and O13T genotypes belonging of red and green areas. The grouping in the same clade of the individuals from Oltina with those from Valea Cismelei shows the similarity between their molecular profiles and allows us to consider that the Cernavoda Plant does not induce harmful influences at the DNA level. In second clade are CNN_{U1}13T and VC13T. Their presence in clade 2 is normal whereas the areas are in the same environmental conditions, under the Cernavoda Plant influence. It can be seen that the similarity of the molecular profile of alfalfa is lower for the inter-populational genotypes compared to that of genotypes in the same location, being lower by 61.5%.

The allocation of ecotypes in the 2014 dendrogram (Figure 4b) is almost similar to the distribution in 2013 dendrogram. The CNN_{U1}14P is locate in the same first position as wll in 2013 starting from the first disjunction being separated from all other branches. $CNN_0$14P and V14P as well as CNN_{U1}14T and VC_S14T are situated in the second claster having a high similarity (Sc-0.6298 respectively Sc-0.6740) but not sufficient high to be consider different. VC14P is grouped in the third clade revealing appropriate molecular profile with O14P and O14T (Sc-0.6519 respectively Sc-0.5414; see Table 7).

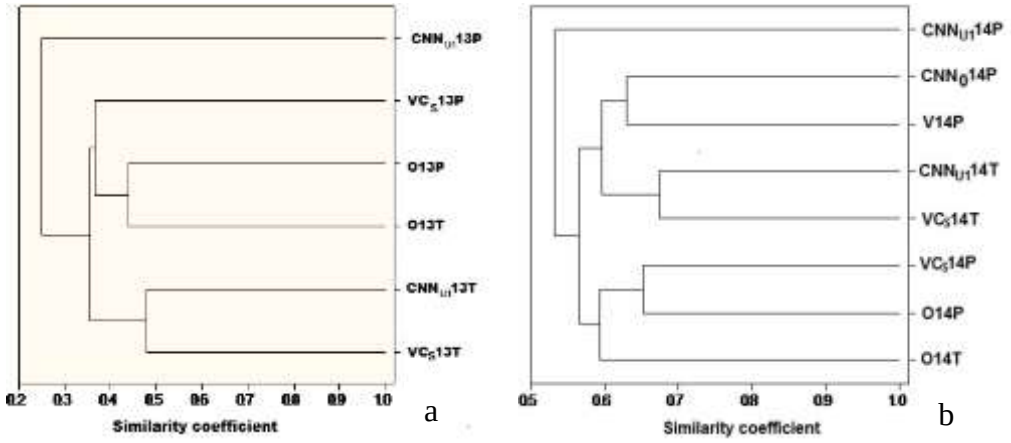


Fig. 4. UPGMA clustering of *Medicago sativa* populations using ISSR primers
(2013) (2014)
Source: Own results.

It should be noted that in 2014 also, the clades include genotypes originating from red areas $CNN_0$14P, CNN_{U1}14T and VC14T with those from green areas V14P, O14P and O14T, emphasizing and in this year their great similarity. The formation of mixed clusters indicates the great likeness between the DNA of the analyzed genotypes and in the same time indicates the unchanged molecular profile of those that grew in red areas. From our point of view, the 2013 and 2014 the Cernavoda Plant emissions do not produced any change in the gene pool of alfalfa plants.

The dendrogram constructed by primer A2-UBC811-UBC816, possesses 7 subclades (Figure 5a) in which are grouped individuals from red areas (four of they i.e. 57.14%); a clade comprises only genotypes from green areas (14.29%); two are 'mixed' having genotypes from green and red ecosystems (28.57%; CNN_{P-TA}15T with $O_1$15P and CNN_{STA}15T with LT15T; Sc-0.8300).

In an appart position are three genotypes that seems to be independent because they are not part of any clade. From the point of separation the CNN_{U1}15T and VC_{S1}15T detachs as the furthest from the rest of the clusters. The genotype $O_3$15T is separated from the clusters, being independent as the two previously mentioned. All the 3 genotypes have a apart disposition are not part of any clade and all comes from autumn plants. In the mixt clades are two completely different genotypes: O15P with CNN_{P-TA}15P (Sc 0.8300), CNN_{STA}15T with LT15T (Sc 0.8300).

Primer products UBC810-A3-A21generated a clear dendrogram but quite similar to the one obtained by the A2-UBC811-UBC816 (Figure 5b).

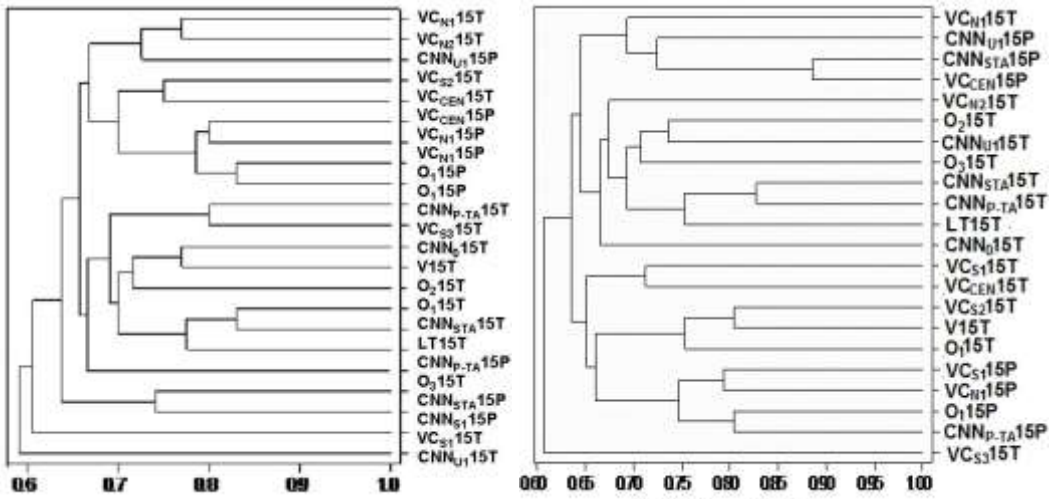


Fig. 5. UPGMA clustering of *Medicago sativa* populations
using A2-UBC811-UBC816 and UBC810-A3-A21 ISSR primers (2015)
Source: Own results.

And this has three genotypes that are not part of any clade. The $VC_{S3}15T$ separates from the very beginning. The $CNN_0 15T$ and $VC_{N2}15T$ branches have an arrangement like to the $CNN_{U1}15T$ and $VC_{S1}15T$ genotypes in the dendrogram generated by A2-UBC811-UBC816. In a similar position was no longer O15T. And this primer has only autumn genotypes.The seven subclades comprise 3 genotypes from the red zone (45.86%) and 4 of them are mixed (57.14%). The large proportion of mixed clades shows once again that in 2015 the DNAs in red and green ecosystems is very similar and indicates the absence of harmful emissions from CNN.

Despite the complexity of the 2015 dendrograms, it was possible to discern aspects that complement the data obtained in previous years.

Both dendrograms grouped on the criterion of similarity at A2-UBC811-UBC816 and UBC810-A3-A2 respectively comprise 7 clades; both have 3 independent genotypes that are not part of any clade; in both dendrograms in different amount poses mixed clades which include the molecular profile of the red and green ecosystems (28.57% and respectively 57.14%).

Dendrograms include cleavages that reflect the evolution of each genotype and the extent to which each has accumulated particularities of their living environment. They provide us with information about the kinship of their genetic background, in our case, between red and green ecotypes.

Mixed clades indicate a similar molecular profile of the nuclear DNA of individuals grown in green areas (Oltina and Vlahi) and those grown in supposedly "polluted" areas (CNN and VC). At the same time, it shows us that the emissions from the Nuclear Plant do not produce changes in the genetic background of alfalfa plants (a dicot species). Alike results were obtained in the case of molecular analysis with *Elymus repens* [(L.) Gould] a monocot specie (work in progress).

3.4. The genetic similarity coefficient

For a clear distinction of the similarity or vice versa to emphasize the variability between the DNAs of alfalfa individuals in red and green ecosystems, a scale was used that includes 5 classes: very small similarity, i.e. very high variability (0-20%), low similarity, i.e. high variability (21-40%), middle similarity or medium variability (41-60%), high similarity i.e. low variability (61-80) and very high similarity i.e. very low variability (81-99%).

Table 5. The genetic similarity coefficient between *M. sativa* populations using ISSR primers (2013)

No.	Population	1	2	3	4	5	6
1	CNN_{U1}13P	**1**	0.2407	0.2667	0.2203	0.2544	0.2583
2	VC_S13P	0.2407	**1**	0.4000	0.3167	0.3534	0.3306
3	O13P	0.2667	0.4000	**1**	0.3419	0.3220	0.4386
4	CNN_{U1}13T	0.2203	0.3167	0.3419	**1**	0.4779	0.3672
5	VC_S13T	0.2544	0.3534	0.3220	0.4779	**1**	0.4262
6	O13T	0.2583	0.3306	0.4386	0.3672	0.4262	**1**

Source: Own results.

The similarity between the CNN_{U1}13P-VC_S13P ecosystems and those from red and green area CNN_{U1}13P-O13P and VCs13P-O13P was small by 24.07%, 26.67% and 40.00%, respectively (see Table 5). The diversity of the DNA profile was high, in both places near (75.93%) and respectively far from the Cernavoda Nuclear Power Plant (73.33%). A middle variability of nuclear DNA was established between CNN_{U1}13T and VC_S13T (52.21%) as well as for the DNA of spring and autumn plants of Oltina (56.14%). Overall, in 2013, for 80% of DNA profiles, there was a low similarity coefficient and only 20% were middle. The highest value of the Similarity coefficient (Sc 0.47779) when it was compared CNN_{U1}13T with VC_S13T, pointing out a like growth medium (see Figure 4a last clade). The similarity of 42.62% for VC_S13T versus O13T is somewhat unpredictable. Valea Cismelei is in the red zone near the Plant, while Oltina is in the green zone outside the influence of CNN. Hypothetically, there should be little resemblance between the DNA of the two ecotypes.

Our results vere confirmed by the analysis of variance (Table 6) that shows a large and significant differences for all populations and seasons. With one exception the F test was small and insignificant (CNN_{U1}13P).

Table 6. Analysis of variance for *Medicago sativa* populations concerning the bands of the ISSR primers (2013)

No.	Populations	Between groups		Within groups		F Test
		SS	DF	SS	DF	
1	CNN_{U1}13P	0.03	1	40.08	179	0.14
2	VC_S13P	1.42	1	42.32	179	6.02*
3	O13P	2.93	1	40.63	179	12.90**
4	CNN_{U1}13T	16.31	1	28.70	179	101.75**
5	VC_S13T	25.04	1	19.90	179	225.19**
6	O13T	10.47	1	34.78	179	53.88**

Source: Own results.

Table 7. The genetic similarity coefficient between *M. sativa* populations using ISSR primers (2014)

No.	Population	1	2	3	4	5	6	7	8
1	CNN_{U1}14P	**1**	0.5304	0.5470	0.5746	0.5470	0.4917	0.5304	0.5083
2	$CNN_0$14P	0.5304	**1**	0.5746	0.5691	0.6298	0.5635	0.5801	0.4917
3	VC_S14P	0.5470	0.5746	**1**	0.6519	0.6243	0.5470	0.5856	0.5414
4	O14P	0.5746	0.5691	0.6519	**1**	0.5525	0.5746	0.5580	0.6464
5	V14P	0.5470	0.6298	0.6243	0.5525	**1**	0.6022	0.6409	0.5525
6	CNN_{U1}14T	0.4917	0.5635	0.5470	0.5746	0.6022	**1**	0.6740	0.5525
7	VC_S14T	0.5304	0.5801	0.5856	0.5580	0.6409	0.6740	**1**	0.6133
8	O14T	0.5083	0.4917	0.5414	0.6464	0.5525	0.5525	0.6133	**1**

Source: Own results.

In 2014, 72.41% of the DNA profile showed an middle similarity and at 27.59% was high. Middle similarity coefficients were obtained for 2 mixed subclades $CNN_0$14P-V14P (Sc 0.6298) and VC_S14P-O14P (Sc 0.6519).

As in 2013, such a similarity was unexpected, because the DNA of the analyzed plants came from red and green areas, a priori considered to be influenced differently by the Cernavoda Nuclear Power Plant.

The similarity between the molecular profiles of the DNA in the two opposing ecosystems indicates the absence of harmful emissions that could come from CNN Plant. There were no changes in the nuclear DNA model of alfalfa plants grown from September 2013 to May 2014 in red or green ecosystems.

The analysis of the variance for 2014 (Table 8) exactly as in 2013 highlighted a single exception in which the difference was insignificant (CNN_{U1}). For 87.5% of the 2014 data, their veracity was confirmed.

Table 8. Analysis of variance for *Medicago* populations concerning the bands of the ISSR primers (2014)

No.	Genotype	Between groups		Within groups		F Test
		SS	DF	SS	DF	
1	CNN_{U1}14P	0.85	1	39.26	179	3.88
2	$CNN_0$14P	5.96	1	36.74	179	29.04**
3	VC_S14P	6.60	1	37.15	179	31.78**
4	O14P	6.93	1	36.63	179	33.88**
5	V14P	12.91	1	32.23	179	71.71**
6	CNN_{U1}14T	10.71	1	34.31	179	55.87**
7	VC_S14T	16.71	1	28.23	179	105.93**
8	O14T	5.61	1	39.64	179	25.35**

Source: Own results.

The primer A2-UBC811-UBC816 (Appendix 1 Table 1.), separated the nuclear DNAs from 22 samples in different classes of similarity: medium (12.02%) large (86.26%) and very large (1.72 %). The greatest similarity was highlighted between the DNA of plants in ecosystems: CNN_{STA}15T with LT15T the $CNN_{P\text{-}TA}$15P with $O_1$15P (83.00%) and VC_{S1}15P with $O_1$15P (80.00%). A wide diversity of alleles was observed at the VC_{S3}15T-$CNN_0$15T genotypes (80.00%).

The similarity coefficients of the BCU810-A3-A21 primers are very similar to those obtained at the A2-BCU811-BCU816 primers (Appendix 2 Table 1.). As expected, the greatest similarity was between the red zone genotypes CNN_{STA}15T-$CNN_{P\text{-}TA}$15T (82.76%), CNN_{STA}15P-VC_{CEN}15P (88.51%), $CNN_{P\text{-}TA}$15P-VC_S15P (80.46%). An unexpected high similarity was revealed by the UBC810-A3-A21 primers for genotypes from the red and green area (CNNP-TA15P-O15P; 80.46%). There are several differences between the two groups of primers that we consider useful to present. Thus, as extreme values, the small and very high similarity coefficients of the primer BCU810-A3-A21 are in proportion higher than those obtained at A2-BCU811-BCU816 (20.09%>12.02% and 2.16%>1.72%); while those with high values are less (77.75% <86.26%).

If for the ecotypes from Valea Cismelei a great similarity is natural (VCS115P-VCN115P = 80.00%), both being part of a large ecosystem under the imminent influence of the Power Plant. But the great similarity of over 80.00% for Valea Cismelei and CNN with Oltina and Lake Tibrin is unnatural. In this situation the similarity are 83.00% at CNN_{STA}15T-LT15T and $CNN_{P\text{-}TA}$15P-$O_1$15P and 80.00% at VC_{S3}15T-$O_3$15T. If the great similarity is debatable in the case of Lake Tibrin

located at 5.51 km from CNN for Oltina located at 33.14 km, the interference of the Cernavoda Plant is excluded, because it is too far to be influenced by Power Plant emissions. The LT15T location is isolated and it is not known if there is a connection through groundwater or from the Danube.

Our data were attested by the analysis of the variance for both groups of primers being large and significant in the vast majority of combinations between and within the target groups (Appendix 1 Table 2 and Appendix 2 Table 2).

The inventory of the similarity coefficients of 2015 seasons, it is found that there are significant differences for the very high similarity for spring ecotypes (4.08%) while between genotypes collected in autumn there is a less uniformity being only 1.26% of them. This finding urges us to consider environmental conditions (TºC, mm, insolation, etc.) as a factor in revealing the molecular differences between ecotypes. The highest number of genotypes with high similarity (Sc>80%) was at the spring ecotypes (62.5%).

At the both primers, A2-UBC811-UBC816 and UBC810-A3-A21 the ADN similarity was high at the red and green areas ecotypes: $CNN_{T\text{-}TA}$15P-O15P (83.00% and 80.46%, respectively) and in VC_{S2}15T-V15T (80.46%).

Table 9. The similarity among alfalfa plants grown in CNN and Oltina ecosystems in two seasons by using primers A2-UBC 811-UBC 816 and UBC 810-A3-A21 (2015)

Ecosystems:		The percent of similarity			
Polluted/ Red	Clean/ Green	A2-UBC 811-UBC 816		UBC 810-A3-A21	
		Spring	Autumn	Spring	Autumn
CNN_{U1}	O_1	72.00	63.00	65.52	63.22
CNN_{STA}	O_1	65.00	65.00	62.07	68.97
$CNN_{P\text{-}TA}$	O_1	83.00	71.00	80.46	72.41
Average		**73.33**	**66.33**	**69.35**	**68.20**

Source: Own results.

The DNA of the alfalfa in the yard of the Cernavoda Power Plant should be different from that of the plants in Oltina, both ecotypes being grown up in differentiated environmental conditions. CNN is located in the red zone and Oltina in the green zone. The molecular profile should have been completely different. However, their similarity is very high (Table 9).

Such a resemblance can only be explained by similar long-term influence of like environmental conditions stress the absence of CNN modifying factors.

In terms of environmental conditions CNN and VC ecosystems are considered to be very like. Consequently, the DNA of alfalfa plants in the Valea Cismelei should be very similar to that of plants of the factory yard (Table 10).

In the 3 groups of genotypes the general similarity coefficient (ScG%) was high highlighting small differences between their DNAs. However, it is worth noting the greater similarity of the DNA from Oltina with that from CNN compared to that of the DNA from VC with CNN (69.30% >64.90%). It would have been natural to have a greater resemblance between similar ecosystems and on the contrary inferior to different ones. The difference of 5% is not big but still allows us to conclude that overall the 3 ecosystems do not differ too much from each other and that CNN Cernavoda does not negatively influence the area.

Table 10. The similarity molecular profile of alfalfa from CNN and VC ecosystems in spring and autumn revealed by A2-UBC 811-UBC 816 and UBC 810-A3-A21 primes (2015)

Ecosystems:		The percent of similarity			
Polluted/ Red	Clean/ Green	A2-UBC 811-UBC 816		UBC 810-A3-A21	
		Spring	Autumn	Spring	Autumn
CNN_{U1}	VC_{CEN}	65.00	56.00	66.67	66.67
CNN_{STA}	VC_{N1}	70.00	62.00	66.67	62.07
$CNN_{P\text{-}TA}$	VC_{S1}	64.00	56.00	80.46	63.22
Average (ScG%)		**66.33**	**58.00**	**71.27**	**63.99**

Source: Own results.

The ^{3}H content in the water collected in the spring campaign are much lower than those of autumn; the average was 6.5±0.44Bq/l respectively 22.1±0.97Bq/l [10]. The air samples for the spring campaign were below the detection limit. The exception is the exclusion zone, Valea Cismelei with 5.81±0.69 Bq/m^3. The measurements from October 2015 were slightly higher, keeping the same exception (8.34±0.73 Bq/m^3). The charge with ^{3}H of the environment cannot be incriminating as a harmful factor of the nuclear DNA. The high similarity of nuclear DNA indicates a similar environment in all ecosystems attested by the quantitative determinations of ^{3}H of water, air and soil.

In general, the literature in this field is limited. The evaluation of the two green and red ecosystems was made by analyzing the quantity and quality of DNA, of electrophoretic bands on gels, the dendrograms clades, variance analysis and similarity between the genotypes of 2013, 2014 and 2015. Each of them revealed more or less obviously the influence of Cernavoda Atomic Power Plant on alfalfa plants as well as on other species.

For Romania, the molecular investigations performed on alfalfa genotypes from the red and green areas of Cernavoda Nuclear Plant influence are singular. In the explored literature we did not find studies related to such aspects. Kamura and co-workers [4] approached the subject tangentially by describing the relatively low ratio of tritium activity (0.37) in the nucleic acid fraction of pea seedlings. They showed also the differentiation of tritium accumulation in biochemical constituents of the plant as well as the preferential incorporation in fats and

proteins. Even alfalfa has been little studied from this point of view [11], although it is a plant used to highlight the environment stress [5].

The general analysis of the structure of the dendrograms in association with the similarity matrix indicates that both the sampling season and the place of origin had an influence on the genetic similarity between the *Medicago* genotypes.

We do not know if those comparative studies have been done to highlight the differences in molecular similarity between spring and autumn genotypes. It was useful to compare the molecular profile of DNA of alfalfa plants in spring and autumn, because new questions arose that should be answered. In the fall, DNA appears to be more complex and the number of allele copies to be higher. The DNA segments released by the proteins that protect them and the histone nucleus (dimers H2A-H2B and tetramers H3-H4) 2 are more susceptible to hybridization with the sequence of primers [13]. High summer temperatures predispose to the relaxation of the histone complex and DNA and thus the naked molecular profile ensures a more efficient hybridization of the primer [1].

Although RAPD and ISSR primers have the disadvantage of labeling homo- and hetero- zygous loci for our aim to emphasize the molecular diversity of individuals in alfalfa populations in green and red ecosystems, the random primers used have been appropriate.

So far, such a finding can only be explained by comparing our data with those reported by the ICSI Ramnicu Valcea research group [10] on the presence of ^{3}H in water, air and soil and its assessment in spontaneous grassy and woody vegetation of the Cernavoda Nuclear Power Plant ecosystems.

The similarity between the molecular profiles of DNA in the two opposing ecosystems indicates the absence of harmful factors that could have been emitted by CNN Plant.

Conclusions

(1) The molecular separation fractions of the 9, 8 and 10 ISSR and RAPD primes used in 3 consecutive years showed a number of 159, 185 and 297 active alleles in the spontaneous alfalfa genotypes in the red and green areas of Cernavoda;

(2) The comparative molecular analyses with RAPD or ISSR primers of spring and autumn plants pointed out a larger molecular diversity at the autumn plants.

(3) The autumn plants seems to be more convenient for that kind of investigations because the DNA profile is more complex, revealed information is more numerous and the monitoring costs would be lower.

(4) We conclude that the variability of the molecular profile of nuclear DNA is associated with environmental variability, especially in intervals spring-autumn rather than with the physical environment created of the emissions from the Cernavoda Power Plant.

(5) Based on the results obtained, we conclude that the emissions from the Cernavoda Power Plant do not produce any change in the genetic background of alfalfa plants.

(6) However, in order to clearly highlight the influence of ^{3}H on the DNA of organisms in the Cernavoda area, several fundamental studies need to be done.

Acknowledgement

This work was supported by SN „*Nuclearelectrica*" SA Cernavoda. Grant BIOTA II no. 71971/30.10.2012.

Authors' contributions

The manuscript was written through contributions of GB and SP; all authors have given approval to the final version of the manuscript; SP made the molecular analysis; the statistical work was performed by GB and SI; the other authors contributed to the data collection.

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Appendix 1 **Table 1**

The genetic similarities coefficients between Medicago sativa populations using A2–UBC811–UBC816

No.	Population	1	2	3	4	5	6	7	8	9	10	11
		12	13	14	15	16	17	18	19	20	21	22
1		**1**	0.770	0.620	0.650	0.620	0.640	0.650	0.640	0.600	0.630	0.660
	$VC_{N1}15T$	0.540	0.620	0.680	0.570	0.730	0.700	0.580	0.630	0.670	0.650	0.600
2		0.770	**1**	0.710	0.680	0.670	0.610	0.640	0.690	0.650	0.720	0.630
	$VC_{N2}15T$	0.530	0.630	0.670	0.580	0.720	0.690	0.630	0.700	0.640	0.660	0.710
3		0.620	0.710	**1**	0.670	0.600	0.640	0.550	0.600	0.580	0.610	0.600
	$VC_{S1}15T$	0.480	0.580	0.560	0.530	0.630	0.620	0.580	0.610	0.610	0.570	0.640
4		0.650	0.680	0.670	**1**	0.690	0.750	0.640	0.630	0.590	0.660	0.590
	$VC_{S2}15T$	0.550	0.730	0.710	0.660	0.660	0.650	0.610	0.720	0.660	0.680	0.690
5		0.620	0.670	0.600	0.690	**1**	0.740	0.670	0.680	0.660	0.670	0.800
	$VC_{S3}15T$	0.640	0.720	0.680	0.690	0.690	0.620	0.620	0.710	0.630	0.650	0.640
6		0.640	0.610	0.640	0.750	0.740	**1**	0.650	0.680	0.640	0.610	0.640
	$VC_{CEN}15T$	0.560	0.660	0.680	0.690	0.670	0.660	0.660	0.730	0.730	0.690	0.700
7		0.650	0.640	0.550	0.640	0.670	0.650	**1**	0.730	0.770	0.620	0.710
	V15T	0.550	0.670	0.730	0.700	0.680	0.630	0.550	0.640	0.660	0.620	0.630
8		0.640	0.690	0.600	0.630	0.680	0.680	0.730	**1**	0.700	0.670	0.700
	$O_1 15T$	0.620	0.680	0.740	0.710	0.690	0.620	0.600	0.670	0.630	0.630	0.600
9		0.600	0.650	0.580	0.590	0.660	0.640	0.770	0.700	**1**	0.670	0.700
	$O_2 15T$	0.560	0.680	0.680	0.710	0.690	0.640	0.580	0.6500	0.670	0.630	0.660
10		0.630	0.720	0.610	0.660	0.670	0.610	0.620	0.670	0.670	**1**	0.690
	$O_3 15T$	0.630	0.650	0.710	0.640	0.620	0.630	0.570	0.640	0.620	0.660	0.590
11		0.660	0.630	0.600	0.590	0.800	0.640	0.710	0.700	0.700	0.690	**1**
	$CNN_0 15T$	0.660	0.680	0.720	0.670	0.710	0.620	0.600	0.6500	0.6500	0.630	0.600
12		0.540	0.530	0.480	0.550	0.640	0.560	0.550	0.620	0.560	0.630	0.660
	$CNN_1 15T$	**1**	0.660	0.700	0.690	0.550	0.580	0.520	0.630	0.610	0.550	0.580
13		0.620	0.630	0.580	0.730	0.720	0.660	0.670	0.680	0.680	0.650	0.680
	$CNN_{STA}15T$	0.660	**1**	0.760	0.830	0.630	0.700	0.660	0.730	0.730	0.690	0.640
14		0.680	0.670	0.560	0.710	0.680	0.680	0.730	0.740	0.680	0.710	0.720
	$CNN_{P\text{-}TA}15T$	0.700	0.760	**1**	0.790	0.670	0.660	0.640	0.730	0.710	0.710	0.620
15		0.570	0.580	0.530	0.660	0.690	0.690	0.700	0.710	0.710	0.640	0.670
	LT15T	0.690	0.830	0.790	**1**	0.660	0.610	0.610	0.760	0.740	0.660	0.650
16		0.730	0.720	0.630	0.660	0.690	0.670	0.680	0.690	0.690	0.620	0.710
	$CNN_1 15P$	0.550	0.630	0.670	0.660	**1**	0.730	0.650	0.720	0.700	0.720	0.710
17		0.700	0.690	0.620	0.650	0.620	0.660	0.630	0.620	0.640	0.630	0.620
	$CNN_{STA}15P$	0.580	0.700	0.660	0.610	0.730	**1**	0.740	0.670	0.690	0.650	0.660
18		0.580	0.630	0.580	0.610	0.620	0.660	0.550	0.600	0.580	0.570	0.600
	$VC_{CEN}15P$	0.520	0.660	0.640	0.610	0.650	0.740	**1**	0.690	0.590	0.670	0.620
19		0.630	0.700	0.610	0.720	0.710	0.730	0.640	0.670	0.650	0.640	0.650
	$VC_{S1}15P$	0.630	0.730	0.730	0.760	0.720	0.670	0.690	**1**	0.800	0.780	0.790
20		0.670	0.640	0.610	0.660	0.630	0.730	0.660	0.630	0.670	0.620	0.650
	$VC_{N1}15P$	0.610	0.730	0.710	0.740	0.700	0.690	0.590	0.800	**1**	0.780	0.790
21		0.650	0.660	0.570	0.680	0.650	0.690	0.620	0.630	0.630	0.660	0.630
	O15P	0.550	0.690	0.710	0.660	0.720	0.650	0.670	0.780	0.780	**1**	0.830
22		0.600	0.710	0.640	0.690	0.640	0.700	0.630	0.600	0.660	0.590	0.600
	$CNN_{P\text{-}TA}15P$	0.580	0.640	0.620	0.650	0.710	0.660	0.620	0.790	0.790	0.830	**1**

Appendix 1

Table 2

Analysis of variance for Medicago sativa populations concerning the bands of A2–UBC811–UBC816

No.	Population	Between groups		Within groups		F Test
		SS	DL	SS	DL	
1	$VC_{N1}15T$	3.121	1	18.639	98	16.41**
2	$VC_{N2}15T$	2.300	1	17.410	98	12.95**
3	$VC_{S1}15T$	0.708	1	22.332	98	3.11
4	$VC_{S1}15T$	2.300	1	17.410	98	12.95**
5	$VC_{S3}15T$	1.756	1	16.484	98	10.44**
6	$VC_{CEN}15T$	1.914	1	17.326	98	10.82**
7	V15T	5.100	1	16.290	98	30.68**
8	$O_1 15T$	2.533	1	16.707	98	14.86**
9	$O_2 15T$	4.767	1	16.993	98	27.49**
10	$O_3 15T$	3.392	1	17.998	98	18.47**
11	$CNN_0 15T$	2.723	1	17.438	98	15.30**
12	$CNN_1 15T$	1.103	1	21.938	98	4.93*
13	$CNN_{STA}15T$	3.033	1	15.207	98	19.55**
14	$CNN_{P\text{-}TA}15T$	5.601	1	12.639	98	43.43**
15	LT15T	3.516	1	15.234	98	22.62**
16	$CNN_1 15P$	2.402	1	14.188	98	16.60**
17	$CNN_{STA}15P$	2.723	1	17.438	98	15.30**
18	$VC_{CEN}15P$	0.000	1	20.160	98	0.00
19	$VC_{S1}15P$	0.478	1	10.832	98	4.33*
20	$VC_{N1}15P$	4.340	1	14.410	98	29.52**
21	O15P	1.284	1	15.306	98	8.22**
22	$CNN_{P\text{-}TA}15P$	1.120	1	16.040	98	6.84*

Appendix 2 **Table 1**

The genetic similarities coefficients between *Medicago sativa* L populations using UBC810–A3-A21 primers

No.	Population	1	2	3	4	5	6	7	8	9	10
		12	13	14	15	16	17	18	19	20	21
1		**1**	0.5977	0.6322	0.6207	0.5862	0.6667	0.5632	0.6092	0.6437	0.6322
	VC_{N1}15T	0.6552	0.6207	0.6322	0.6552	0.6322	0.7356	0.7126	0.6782	0.6092	0.5862
2		0.5977	**1**	0.5977	0.6322	0.5517	0.5632	0.5747	0.6667	0.6552	0.6897
	VC_{N2}15T	0.6667	0.7011	0.6667	0.6667	0.6437	0.6782	0.7471	0.6667	0.6897	0.6207
3		0.6322	0.5977	**1**	0.5517	0.6552	0.7126	0.5862	0.7011	0.5977	0.6322
	VC_{S1}15T	0.6552	0.6667	0.6322	0.6552	0.6552	0.5747	0.5747	0.6092	0.6322	0.6322
4		0.6207	0.6322	0.5747	**1**	0.6207	0.6322	0.8046	0.7586	0.6552	0.6667
	VC_{S2}15T	0.6897	0.6782	0.7586	0.6207	0.6667	0.6322	0.6552	0.7126	0.6897	0.6437
5		0.5862	0.5517	0.6552	0.6207	**1**	0.6207	0.6552	0.6782	0.6437	0.5632
	VC_{S3}15T	0.5862	0.6667	0.6552	0.6552	0.6322	0.5057	0.5287	0.5632	0.5632	0.6322
6		0.6667	0.5632	0.7126	0.6322	0.6207	**1**	0.7126	0.7356	0.6322	0.6897
	VC_{CEN}15T	0.6667	0.6092	0.5977	0.6207	0.7126	0.6322	0.6092	0.6897	0.6437	0.6207
7		0.5632	0.5747	0.5862	0.8046	0.6552	0.7126	**1**	0.7471	0.6207	0.6782
	V15T	0.6322	0.6667	0.6552	0.5862	0.7011	0.5747	0.5747	0.6782	0.6322	0.6092
8		0.6092	0.6667	0.7011	0.7586	0.6782	0.7356	0.7471	**1**	0.6667	0.7011
	$O_1$15T	0.6322	0.6897	0.7241	0.6782	0.6552	0.5747	0.5747	0.6782	0.6782	0.6322
9		0.6437	0.6552	0.5977	0.6552	0.6437	0.6322	0.6207	0.6667	**1**	0.7126
	$O_2$15T	0.7356	0.7011	0.7126	0.6897	0.6667	0.5632	0.5862	0.5977	0.6667	0.5747
10		0.6322	0.6897	0.6322	0.6667	0.5632	0.6897	0.6782	0.7011	0.7126	**1**
	$O_3$15T	0.7011	0.7356	0.6552	0.7011	0.7241	0.6437	0.6437	0.6092	0.6092	0.5862
11		0.6207	0.6092	0.5977	0.5632	0.5977	0.5862	0.5977	0.5517	0.6782	0.6667
	$CNN_0$15T	0.6207	0.7011	0.7126	0.6667	0.6667	0.5862	0.6552	0.5287	0.5977	0.5517
12		0.6552	0.6667	0.6552	0.6897	0.5862	0.6667	0.6322	0.6322	0.7356	0.7011
	$CNN_1$15T	**1**	0.6667	0.6782	0.7011	0.6782	0.6207	0.6667	0.7011	0.7011	0.6322
13		0.6207	0.7011	0.6667	0.6782	0.6667	0.6092	0.6667	0.6897	0.7011	0.7356
	CNN_{STA}15T	0.6667	**1**	0.8276	0.7816	0.6437	0.6092	0.6552	0.6667	0.7126	0.6437
14		0.6322	0.6667	0.6322	0.7586	0.6552	0.5977	0.6552	0.7241	0.7126	0.6552
	CNN_{P-TA}15T	0.6782	0.8276	**1**	0.7241	0.6782	0.6667	0.6897	0.6322	0.7011	0.6092
15		0.6552	0.6667	0.6552	0.6207	0.6552	0.6207	0.5862	0.6782	0.6897	0.7011
	LT15T	0.7011	0.7816	0.7241	**1**	0.6322	0.6207	0.6207	0.6092	0.6552	0.5862
16		0.6322	0.6437	0.6552	0.6667	0.6322	0.7126	0.7011	0.6552	0.6667	0.7241
	$CNN_1$15P	0.6782	0.6437	0.6782	0.6322	**1**	0.7356	0.7126	0.6322	0.6322	0.6552
17		0.7356	0.6782	0.5747	0.6322	0.5057	0.6322	0.5747	0.5747	0.5632	0.6437
	CNN_{STA}15P	0.6207	0.6092	0.6667	0.6207	0.7356	**1**	0.8851	0.7126	0.6667	0.6207
18		0.7126	0.7471	0.5747	0.6552	0.5287	0.6092	0.5747	0.5747	0.5862	0.6437
	VC_{CEN}15P	0.6667	0.6552	0.6897	0.6207	0.7126	0.8851	**1**	0.6897	0.6667	0.6437
19		0.6782	0.6667	0.6092	0.7126	0.5632	0.6897	0.6782	0.6782	0.5977	0.6092
	VC_{S1}15P	0.7011	0.6667	0.6322	0.6092	0.6322	0.7126	0.6897	**1**	0.7931	0.7241
20		0.6092	0.6897	0.6322	0.6897	0.5632	0.6437	0.6322	0.6782	0.6667	0.6092
	VC_{N1}15P	0.7011	0.7126	0.7011	0.6552	0.6322	0.6667	0.6667	0.7931	**1**	0.7011
21		0.5862	0.6207	0.6322	0.6437	0.6322	0.6207	0.6092	0.6322	0.5747	0.5862
	O15P	0.6322	0.6437	0.6092	0.5862	0.6552	0.6207	0.6437	0.7241	0.7011	**1**
22		0.6667	0.6092	0.6437	0.6782	0.5747	0.7011	0.6437	0.6437	0.6092	0.6667
	CNN_{P-TA}15P	0.7356	0.5862	0.5977	0.5517	0.6897	0.6552	0.6782	0.8046	0.7586	0.8046

Appendix 2

Table 2

Analysis of variance for Medicago sativa populations concerning the bands of UBC810–A3-A21 primers

No.	Population	Between groups		Within groups		F Test
		SS	DF	SS	DF	
1	$VC_{N1}15T$	3.903	1	14.327	85	23.16**
2		1.718	1	16.099	85	9.07**
3	$VC_{N2}15T$	1.249	1	17.739	85	5.99*
4		0.000	1	14.851	85	0.00
5	$VC_{S1}15T$	0.399	1	18.590	85	1.82
6		3.653	1	14.967	85	20.75**
7	$VC_{S2}15T$	1.006	1	17.224	85	4.97*
8		0.435	1	14.967	85	2.47
9	$VC_{S3}15T$	0.134	1	13.544	85	0.84
10		2.260	1	14.177	85	13.55**
11	$VC_{CEN}15T$	1.397	1	17.224	85	6.90*
12		0.454	1	12.604	85	3.06
13	V15T	0.524	1	14.327	85	3.11
14		0.005	1	11.742	85	0.04
15	$O_1 15T$	2.618	1	14.762	85	15.07**
16		3.050	1	13.387	85	19.37**
17	$O_2 15T$	5.748	1	12.872	85	37.96**
18		5.212	1	12.604	85	35.15**
19	$O_3 15T$	3.050	1	13.387	85	19.37**
20		1.313	1	14.090	85	7.92**
21	$CNN_0 15T$	1.006	1	17.224	85	4.97*
22		2.830	1	14.090	85	17.07**