The Journal of Animal & Plant Sciences, 22(2): 2012, Page: 369-375
ISSN: 1018-7081

DETERMINATION OF GENETIC RELATIONSHIP IN TURKISH CHICKPEA (*CICER ARIETINUM* L.) GENOTYPES USING SSR MOLECULAR MARKERS AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

This study aimed to determinate the genetic relationship among 29 chickpea (*Cicer arietinum* L.) genotypes in Turkey using SSR markers. A total of 13 SSR primers were used for PCR amplifications, but the study was conducted with the data obtained from 10 SSR primers that yielded good results. PCR products were separated by the Beckman Coulter CEQ-8000 genetic analysis system with capillary electrophoresis and peak values of each primer were scored as presence (1) or absence (0) for all genotype. The dendrogram was constructed based on the genetic similarity coefficient using the UPGMA (unweighted pair group with arithmetic average) method with the computer software NTSYS-pc. Also, polymorphism rates, heterozygosity and polymorphic informaiton content were calculated using statistical formula. A total of 134 alleles were obtained from 10 primers and 122 of them were turned out to be polymorphic and the allele numbers obtained with SSR primers varied between 4 and 28. Heterozygosity also ranged between 0.23 and 0.42. Polymorphic information contents (PIC) varied between 0.70 and 0.89. Genetic similarity coefficients changed between 0.45 and 0.87. Chickpea genotypes identified among the very high level of similarity to that obtained from the dendogram branches intertwined. As a result, Turkish chickpea genotypes can be expressed in a very narrow genetic base.

Key words: Chickpea, *Cicer arietinum* L., SSR, capillary electrophoresis, genetic relationship.

INTRODUCTION

The genus *Cicer* includes very important species of the *Papilionaceae* subfamily of the *Leguminosae* family (Akçin 1988). The genus *Cicer* includes a total of 43 species which involves 9 annual species containing cultured chickpea, 33 perennial species and 1 unclassified species (Sethy *et al*. 2006a). Among these groups, the only variety which has economic importance is *Cicer arietinum* L.. It is a cultivated chickpea and has a genome of 750 Mbp in size. *C. arietinum is a* self pollinated diploid legume with a basic chromosome number of 8 (Sethy *et al*. 2006b).

The origin of chickpea is the south-eastern Turkey and northern Syria (Güneş *et al*. 2006). Chickpea had been found in the ruins dating back to 8000 B.C. in south-eastern Turkey and in the northern Syria (Diamond 2006). Although it is extensively adaptable and cultivated in vast areas across the world, biotic and abiotic stress factors such as micronutrient deficiencies, cold, drought, salinity and leaf diseases limit chickpea yield (Mahmoudi *et al*. 2007). Genetic variation is very limited in *Cicer arietinum*, and this causes difficulty in breeding efforts aimed at developing new species resistant to these stress conditions (Mantri *et al*. 2007). Genetic polymorphism is extremely low in cultured chickpeas so that developing new varieties is difficult through classical breeding methods. In case of that, in recent years molecular markers have been used in breeding programs (Winter *et al*. 1999).

Buhariwalla *et al*. (2005) expressed that the genetic base has narrowed in cultivated chickpea due to limited adaptability of wild ancestors and the effects that emerge during breeding, that the rate polymorphism is higher than others in studies where SSR markers have been used, and that this information can be used in breeding programs based on markers. Lichtenzveig *et al*. (2005) stated that when it is compared with other marker types in many plants with low polymorphism, SSR or microsatellite markers are more effective in overcoming this problem. Kraic *et al*. (2002) stated that according to the results of polymorphic information content, difference index and similarity probabilities, SSR applications are valuable in the management of barley, soybean and chickpea genetic sources. The present study aims to determine the genetic relationship levels of some chickpea genotypes in Turkey through SSR molecular markers and to contribute to Chickpea breeding programmes involving marker-assisted breeding of the species.

MATERIALS AND METHODS

Material: A total of 29 chickpea genotypes, including 20 registered chickpea varieties (1-20) in Turkey, 6 lines

(21-26) and 3 local chickpea populations (27-29) were used in the study (Table 1).

Table 1. The Chickpea genotypes used as a plant material in SSR analyses

No	Genotype	No	Genotype	No	Genotype
1	Canıtez-87	11	Aydın-92	21	52. Hat (breeding line)
2	Yaşa-05	12	Sarı-98	22	80. Hat (breeding line)
3	Işık-05	13	Cevdetbey-98	23	65. Hat (breeding line)
4	Akçin-91	14	Damla-89	24	46. Hat (breeding line)
5	Gökçe	15	Çağatay	25	1. Hat (breeding line)
6	Küsmen-99	16	Gülümser	26	14. Hat (breeding line)
7	Uzunlu-99	17	Aziziye	27	Kadınhanı (local population)
8	Er-99	18	ILC-482	28	Seydişehir (local population)
9	İzmir-92	19	Diyar-95	29	Beyşehir (local population)
10	Menemen-92	20	İnci		

Determination of DNA Isolation and Concentration: The youngest leaves of 20-day old chickpea seedlings grown in a greenhouse were used for DNA isolation. Each genotype was represented with 10 individuals in isolation, and equal numbers of leaf samples (~20 mg, total 200 mg) were taken from these randomly selected 10 plants, mixed and then DNA isolation was performed using the modified 2xCTAB method. Concentrations of DNAs were determined on the spectrophotometer (Nanodrop, model ND-100) after isolation. DNAs were diluted to 20 ng μl^{-1}, which is the study concentration. Equal amounts of the samples from prepared concentration were passed into the 1 % agarose gel (1xTBE buffer) and equivalent of the concentration were observed.

DNA Amplifications Using the SSR Molecular Marker Technique: Thirteen SSR primers which were determined as a result of a review of literature were used for DNA amplifications using the SSR molecular marker technique (Winter 1999, Sety *et al*. 2006a, Sety *et al*. 2006b, Lichtenzveig *et al*. 2005) (Table 2). Analyses of universal primers marked with fluorescence and primer tailing were performed in order to reduce the cost of the study (Bandelj *et al*. 2004). Fluorescence WELL-RED D4 marked (blue) universal M13 primer, which is compatible with the Beckman Coulter CEQ-8000 Genetic Analysis System, was added to the PCR reaction mixture at the level of 2.5 pmol in order to obtain peaks at capillary electrophoresis (Guo *et al*. 2003).

After the reactions were optimized through pre-tests, 2 µl DNA (20 ng μl^{-1}) and 23 µl reaction mixture [2.5 µl x10 PCR buffer solution (Fermentas), 2.5 µl 25 mM $MgCl_2$ (Fermentas), 0.4 µl 25 mM dNTP (Fermentas), 0.3 µl 500 U Taq DNA Polymerase (Fermentas), 0.25 µl 50 pmol μl^{-1} primer (from each of the forward and reverse primers), 0.1 µl 50 pmol μl^{-1} fluorescence marked primer (D4) and 16.7 µl PCR water] was performed in 40 cycles on the Eppendorf PCR device in the form of Touchdown PCR by considering the Tm temperatures of the primers.

The PCR reaction was carried out in a touchdown fashion with a first denaturation at 94°C for 3 min, followed by 6-15 cycles of; (1) denaturation at 94°C for 1 min, (2) annealing at above 5-6 °C of temperature melting (Tm) for 1 min, and (3) extension at 72°C for 45 sec, with the annealing temperature being reduced by 0.5°C per cycle. This procedure was followed by 25-34 cycles of; (1) denaturation at 94°C for 1 min, (2) annealing at Tm for 1 min, and (3) extension at 72°C for 45 sec, and a final extension at 72°C for 10 min.

Each 0.5 µl PCR product, after the addition of SLS (Sample Loading Solution) containing 25 µl Hi-Di formamide and S-400 DNA Standard (Beckman Coulter p/n 608098), was separated through capillary electrophoresis in the Genetic Analysis System with FRAG-3 method using 33 cm DNA separation capillary (Beckman Coulter p/n 608087). Marker alleles were defined through "default" calibration using CEQ fragment analysis program.

Analysis of PCR amplification analysis data: Polymorphism rates (PR) of the SSR primers used in the study were calculated by dividing the polymorphic allele numbers obtained from the primers by the total number of alleles and then multiplying them with 100.

Polymorphism information contents (PIC) of the SSR primers used in the study were determined with the help of PIC= 1 - Σ Pi^2 (Smith *et al*. 1997). According to this, first, the peak values that were obtained from capillary electrophoresis and fragment analysis were determined. Alleles were scored identifying them as "present" (1) and "absent" (0) in the genotypes and then their frequencies were calculated separately. The "Pi" in the formula is the frequency of "i." allele.

The heterozygosity rates (H) of the SSR primers used in the study were calculated using the basic equation used in the population genetics according to H= $1-p^2-q^2$ (Nei 1972). Here, "p" stands for existence number of "i." allele, whereas "q" represents the number for absence of i. allele.

Table 2. The SSR primers was used in this study*

Primer	Primer sequence (5'→3')	Length (bp)	GC (%)	Tm (°C)
GA6_Cicer_F	5'- **GT GCA GAG CAT CAT GC**ATT TTT CTC CGG TGT TGC AC -3'	36	50.0	77.0
GA6_Cicer_R	5'- AAA CGA CAG AGA GTG GCG AT -3'	20	50.0	57.3
GA26 Cicer_F	5'- **GT GCA GAG CAT CAT GC**GAT GCT CAA GAC ATC TGC CA -3'	31	51.6	74.0
GA26 Cicer_R	5'- TCA TAC TCA ACA AAT TCA TTT CCC -3'	24	33.3	55.9
GAA60_Cicer_F	5'- **GT GCA GAG CAT CAT GC**TTG GTT TGC AAA TTG TTC TTC -3'	37	43.2	74.0
GAA60_Cicer_R	5'- AAG TCC ATT GAA GTG TCG CC -3'	20	50.0	57.3
TS29_Cicer_F	5'- **GT GCA GAG CAT CAT GC**AAC ATT CAT GAA CCT ACC TCA ACT TA -3'	42	42.9	77.0
TS29_Cicer_R	5'- CCA TAT ATG AGT ACA CTA CCT CTC GG -3'	26	46.2	63.2
CrtSSR2_F	5'- **GT GCA GAG CAT CAT GC**GAG GTT TGG TGA AGG TAT GA -3'	36	50.0	77.0
CrtSSR2_R	5'- GTG CTG GTT CTT CTT CGT TA -3'	20	45.0	56.0
CrtSSR47_F	5'- **GT GCA GAG CAT CAT GC**TGA GGC CTA AGA GTA CCA AA -3'	36	50.0	77.0
CrtSSR47_R	5'- TCT CAT CAG GAA CAA CAA CA -3'	20	40.0	54.0
NCPGR33_F	5'- **GT GCA GAG CAT CAT GC**ACA TCT TGA AGT GCC CCA AC -3'	36	52.8	78.0
NCPGR33_R	5'- TGC AAG CAG ACG GTT ACA AG -3'	20	50.0	78.0
NCPGR50_F	5'- **GT GCA GAG CAT CAT GC**ATG ATG GAT TTT CGG AAT GT -3'	36	44.4	74.0
NCPGR50_R	5'- AAA AAT GCT GGA AGG AAC TG 3'	20	40.0	54.0
NCPGR51_F	5'- **GT GCA GAG CAT CAT GC**CAT AAT GCA AGG GCA ATT AG -3'	35	45.7	74.0
NCPGR51_R	5'- CTC TTA TCT TCA TGT TGC CG -3'	20	45.0	56.0
NCPGR53_F	5'- **GT GCA GAG CAT CAT GC**CCC TCC TTC TTG CTT ACA AA -3'	35	48.6	75.0
NCPGR53_R	5'- TAA TGG TGA ACG AAT CAT GG -3'	20	40.0	54.0
NCPGR60_F	5'- **GT GCA GAG CAT CAT GC**AGA AAT CAC AAA CCT CTT CG -3'	36	47.2	75.0
NCPGR60_R	5'- GCT TGG ATC TTC AAA ACT TG -3'	20	40.0	54.0
H1B09_F	5'- **GT GCA GAG CAT CAT GC**GGT TTC ATG ACC TGC ACC TA -3'	36	52.8	78.0
H1B09_R	5'- AAG AAC CGA AAA CAC TTG TGA -3'	21	38.1	55.0
H1B17_F	5'- **GT GCA GAG CAT CAT GC**ATT CGA GGT GGT ACC TCT AGT GA -3'	39	51.3	79.0
H1B17_R	5'- GAG GAA CCG ACG ATG TAT CTA TT -3'	23	43.5	61.0

* Primer sequence of M13 was indicated in bold and underlined.

Taking into consideration, the peak values that the products which were obtained as a result of PCR amplifications performed through SSR primers formed in the Beckman Coulter CEQ-8000, turned into numerical values as present (1) / absent (0) and using the NTSYS-pc 2.1 genetic analysis program (Rohlf 2004), dendogram and genetic relationship coefficients indicating the genetic relationships of chickpea genotypes used in the experiment were obtained. The similarity coefficients of genotypes were formed according to the SM (Simple Matching) coefficient using NTSYS-pc 2.1.

RESULTS AND DISCUSSION

Although 13 SSR primers were used in the study, 3 primers (TS-29, GAA-60, H1B17) were produced unscorable results and they were excluded from the study. The study was conducted with the data obtained from 10 SSR primers that yielded good results (Figure 1).

Due to the fact that DNA isolation was performed collectively from 10 individuals, large numbers of alleles were observed in each genotype and therefore prominent alleles that were frequently encountered in each genotype were evaluated. The increase in the number of alleles in collective DNA isolations was a normal phenomenon when the sources of allels of the individual were from the mother and father. Wang *et al*. (2007), Yao *et al*. (2007), Hwang *et al*. (2008) and Khan *et al*. (2009) were preferred collective DNA isolation, as we did in study, in wheat, corn, wild soybean and cotton, respectively. They scored allele magnitudes belonging to the PCR products which they obtained with SSR primers on the basis of whether they were present (1) or absent (0) and completed genetic relationship studies successfully. The same researchers generally made their analyses using the NTSYS-2.1 pc program. The method used in this study is in accordance with the literature.

The allele range, allele number, polymorphic allele number, polymorphism rate, heterozygosity, polymorphism information content and separation efficiency values that were obtained as a result of an evaluation of the data belonging to the products acquired from PCR amplifications performed with SSR primers were given separately in Table 3.

The allele numbers obtained with SSR primers varied between 4 and 28, and the highest number of allel was obtained from the GA-26 primer (28), whereas the lowest number of allel was obtained from the SSR-2 primer (4). A total of 134 alleles were obtained from 10 primers and 122 of them were turned out to be polymorphic. When an evaluation is performed in terms of polymorphic alleles,

it can be seen that potentials of primers used in the study for forming polymorphic bands are high. It was determined that all of the primers except for PGR-60 (13/20)(65%), PGR-51 (7/10)(70%), SSR-47 (4/5)(80%) and SSR-2 (3/4)(75%), all alleles were polymorphic (100%).

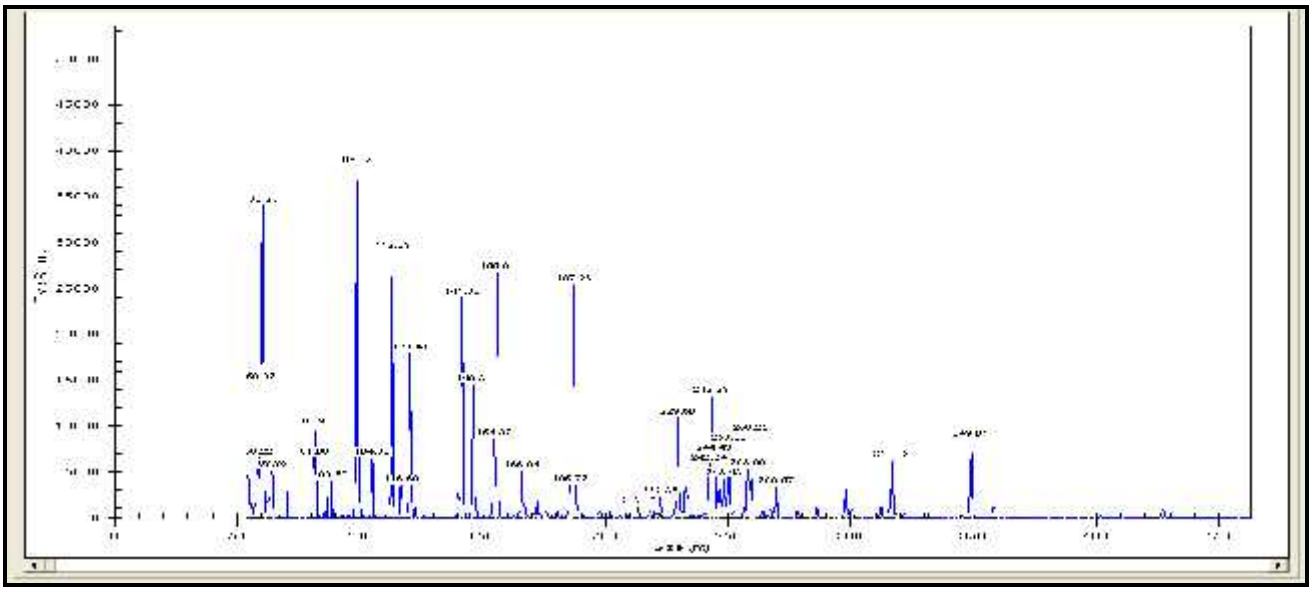

Figure 1. The peaks form GA-26 primary as a result of capillary electrophoresis analysis of fragments obtained from the Gülümser cultivar.

Table 3. The result of an evaluation of the data belonging to the products acquired from PCR amplifications performed with SSR primers*

Primer	Allel range (bp)	Allel number	Polimorfic allel	Polymorphism (%)	Heterozygosity rate (H)	Polymorphic Information Content (PIC)
GA-6	114-410	9	9	100	0.28	0.73
H1B09	150-274	18	18	100	0.33	0.79
PGR-60	172-192	20	13	65	0.24	0.73
PGR-51	141-285	10	7	70	0.23	0.89
GA-26	114-258	28	28	100	0.34	0.89
PGR-53	144-286	13	13	100	0.42	0.70
SSR-47	143-212	5	4	80	0.33	0.85
PGR-33	153-299	8	8	100	0.38	0.79
PGR-50	155-305	19	19	100	0.32	0.74
SSR-2	120-285	4	3	75	0.25	0.86
Total		**134**	**122**		**3.13**	**7.97**
Mean		**13.4**	**12.2**	**89**	**0.31**	**0.80**

* All alleles were taken into account in the calculations obtained from the primers. PIC values, the resulting of value have determined by dividing the total number of alleles.

It was found that SSR primers used in the study exhibited heterozygosity within the range of 0.23-0.42. If the average heterozygosity value of 0.31 considered, it can be commented that heterozygosity is low in chickpea genotypes or exhibit similarities in terms of the primer zones that were investigated. Banerjee *et al*. (2001) pointed out that presence of polymorphic locus in chickpea is limited.

Polymorphic information contents (PIC) of the 10 SSR primers that were used varied between 0.70 and 0.89. Average PIC was determined to be 0.80, while the higher ones obtained from PGR-51 and GA-26 primers (0.89), and the lowest value was obtained from PGR-53 primer (0.70). Upadhyaya *et al*. (2008) used SSR markers on 300 chickpea accessions to determine genetic structure and difference and they stated that the PIC value was varied between 0.47 and 0.97 and the mean value was

0.85. When an overall evaluation is made, it was observed that PIC values of the primers used in the study were quite high. Lichtenzveig *et al*. (2005) stated that SSR markers were more effective in overcoming this problem in many plants with low polymorphism.

Although, PIC values were high, heterozygosity values were generally low. The heterozygosity values of the primers used in the study varied between 0.23 to 0.42, and their mean value was determined to be 0.31. Heterozygosity values were high in primers where polymorphism was found to be high.

It can be concluded that genetic information is quite similar in chickpea genotypes. Chowdhury *et al*. (2002) stated that a high level of homogeneity formed in chickpea varieties and culture varieties/breeding lines having the same genetic base exhibited close similarity. Mantri *et al*. (2007), pointed out that although cultured chickpeas had high morphological variation, genetic variation was very little. The results are in conformity with the information in the relevant literature.

Genetic similarity coefficients among the chickpea genotypes determined through an analysis of amplification products obtained via SSR analyses on NTSYS-2.1 pc program varied between 0.45 and 0.87. When chickpea genotypes were examined in terms of genetic similarity, the genotypes that are closest to one another were Beyşehir and Seydişehir (0.87), Küsmen-99 and Akçin-91 (0.81), Seydişehir and Işık-05 (0.81), Seydişehir and line 14 (0.81), and Canıtez-87 and Yaşa-05 (0.80). Beyşehir and ILC-482 (0.45), ILC-482 and Işık-05 (0.48), Menemen-92 and Yaşa-05 (0.49), line 52 and Yaşa-05 (0.49), line 14 and İnci (0.49) were the genotypes with the least similarity and were farthest from one another.

When the similarity coefficients of other genotypes were examined, it was observed that most of them take a value between 0.60 and 0.69. It may be concluded that the genetic similarity is high among the genotypes. The genetic relationship dendogram given in Figure 2 reveals this situation clearly.

Genetic relationship dendogram were obtained according to the UPGMA method using the NTSYS-2.1 pc program (Figure 2).

When the genetic relationship dendogram, given in Figure 2 is examined, it is seen that chickpea genotypes fall into two groups with two major branching. While, the first basic group involves almost all of the varieties, lines and local populations, the second basic group contains only ILC-482 variety. ILC-482 was a variety of ICARDA origin registered by Southeastern Agricultural Research Institute. Therefore, it was likely to be considered an external group. Nevertheless, its similarity to the groups including all of the other genotypes is at the level of 0.56. This high level of similarity may be considered to be normal, due to the shrinkage in genetic base even if it is foreign origin.

The first basic group was divided into two main subgroups within itself. While, the first of these main groups again consist of almost all of the genotypes, only İzmir-92, Menemen-92 and İnci varieties were separated from the others and form the 2nd main sub-group. İzmir-92 and Menemen-92 varieties were developed by Aegean Agricultural Research Institute and the similarity level in the dendogram was at the level of 0.75.

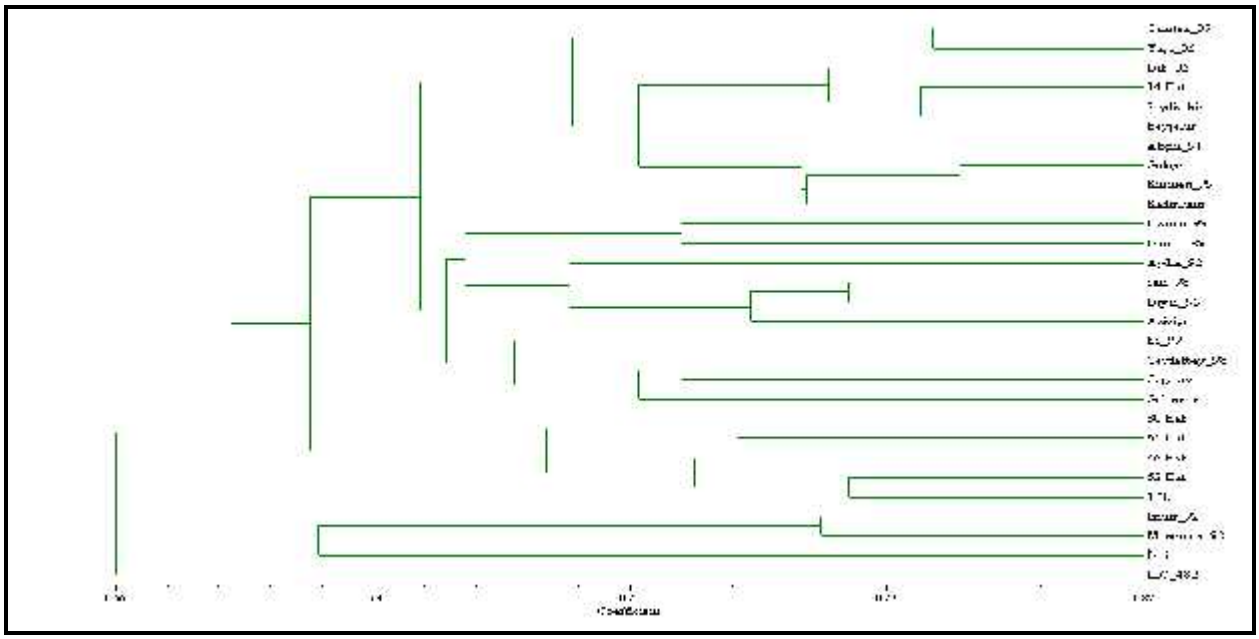

Figure 2. Genetic relationship dendogram of chickpea genotypes according to SSR molecular marker technique obtained by using NTSYS-2.1 pc.

The first main group further tends to group in two branches. In branching at this level, the differences between genotypes begin to become more evident. It was determined that one of the group consisted of the lines that we used in the study and that similarity levels of the lines were at the level of 0.70-0.79. The fact that the lines exhibit a high level of similarity among themselves may be interpreted to be a result of narrow genetic base. Moreover, these lines have high level of similarity to the registered varieties. A new branching also occurred within the other group. Canıtez-87, Yaşa-05, Işık-05, line 14, Akçin-91, Küsmen-99 and Kadınhanı local population were in the same group. Seydişehir and Beyşehir local populations have a similarity level of 0.87 and were the closest genotypes to each other in the dendogram. When this group was evaluated on the basis of the source institute, it was observed that it was composed of Anatolian Agricultural Research Institute, Central Research Institute for Arable Plants and local populations. Uzunlu-99, Damla-89, Aydın-92, Sarı-98, Diyar-95, Aziziye, Er-99, Cevdetbey-98, Çağatay and Gülümser genotypes also form a group among themselves.

Although, the genotypes in this group were of different institute origins and group were not clear as in the case of lines, it is seen that when they were evaluated in groups of 2 and 3, the genotypes which bred in the same institute, were closer to each another. It is apparent that the institutes far from each other were grouped together in the dendogram may be a result of the material exchange between the institutes. It is stated by the institutes, which registered the samples, that most of the chickpea genotypes were suitable for farming in all areas. This may be evaluated as they have a lot of common properties. Upadhyaya *et al*. (2008) stated that an extensive germplasm collection in chickpea as in other legumes and international breeding programs are implemented although genetic base is fairly limited.

Although, SSR marker technique is a molecular technique having a high distinctive capacity, the similarity found among chickpea genotypes is at a very high level. Therefore, interpenetrating branching is very common in the dendogram. However, all the genotypes could be distinguished from one another using the primers.

Conclusion: As a result of scoring, it appeared that polymorphism was quite low in the chickpea genotypes that were used in our study as research material.

The genetic similarity coefficients among the chickpea genotypes, which were determined as a result of the analysis of amplification products obtained from SSR analyses on the NTSYS-2.1 pc program, varied between 0.45 and 0.87. Although, SSR is a molecular marker technique with high separation efficiency. The similarity determined among the chickpea genotypes were quite high. Similarities were at the level of 0.60 and above. The fact that the lines exhibit high similarity among themselves may be interpreted to be a result of limited genetic base. Moreover, another interesting thing is that these lines and local populations are closely related to registered (certified) varieties. Common properties may be considered to be quiet high.

The need to evaluate the variation of the sources and develop new varieties, compatible with today's quality criteria that have high yield, quality and resistant to disease, has arisen for the plant which is native to Turkey. Our study encompasses almost all of the registered varieties that may guide molecular selection efforts or breeding programs where wild varieties are used as parents of the registered varieties.

An edible legume is very important among the arable plants in terms of area of growing. One of the fundamental problems with the chickpea is that it has a limited genetic base during the process of cultivation. This causes serious problems in terms of breeding and difficulty in improving nutrition content or increasing tolerance to stress. DNA marker technologies that are used together with breeding programs help to increase the nutrition quality where plant nutrition and molecular studies intersect and also lead to the development of genotypes resistant to unfavourable soil conditions and widespread use of authentic technologies such as marker-assisted selection (MAS).

Acknowledgments: This study is part of a PhD thesis by Emine Atalay and was supported by the Selcuk University Scientific Research Fund.

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