

## ANALYSIS OF GENETIC DIVERSITY OF SUGAR BEET GENOTYPES USING RANDOM AMPLIFIED POLYMORPHIC DNA MARKER

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Plant breeding programs are formulated based on the diversity and selection of superior quantitative and qualitative traits. Hence, assessment of genetic diversity is the first step of every plant breeding program. In this regard, use of new methods for studying genetic diversity seems necessary. In the present study, the genetic diversity of thirty sugar beet genotypes was determined using Random Amplified Polymorphic DNA (RAPD) marker. Following the DNA extraction and optimization of experiment conditions, of the 40 primers under study, 10 primers that induced polymorphism and produced good and clear bands in the genotypes of sugar beet were randomly selected. Statistical calculations were carried out based on the Jaccard similarity coefficient and UPGMA-based grouping in the NTSYS software (version 2.02). The amplitude of the multiplied bands varied between 100 and 3000 of alkaline pair. The polymorphism of all primers was 82.33% within the similarity limit. The Cophenetic coefficient for the similarity matrix and the resulting curve was obtained to be  $r=0.75$ . Genotypes 4 and 18 with a similarity coefficient of 0.91% demonstrated the highest similarity while genotypes 21 and 15 with a similarity coefficient of 0.63% showed the lowest similarity. Of the primers in use, the OPB-18 primer produced 12 bands (the highest number of bands) and the OPA-09 primer produced 5 bands (the lowest number of bands). Cluster analysis also confirmed the results obtained from the profiles produced in the genetic differentiation of cultivars under study as well as the correlations resulting from the Jaccard similarity coefficient. Finally, genotypes were categorized into 13 groups based

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on the results and resulting dendrogram. Results of the cluster analysis performed using the Jaccard similarity coefficient revealed the genetic diversity among genotypes that emphasize on efficiency of selection in sugar beet genotypes.

*Key words:* Beta vulgaris, RAPD, genetic analysis, cluster analysis, UPGMA

#### INTRODUCTION

Sugar beet, scientifically known as *Beta vulgaris* L., is a biennial plant of the order Poriferans that is cultivated as an annual plant. Sugar beet lacks stem in the course of its growth and is seen in the form of large horizontal and vertical leaves. The maturity required for production of sugar varies between 6 and 9 months. *Beta vulgaris* L. is one of the two important sources of production of sugar in the world (AMIRI, 2003). About one fourth of the world sugar demand in moderate regions, where sugarcane is not grown, is supplied by sugar beet (DRAYCOTT, 2006). The sugar beet cultivated in mountainous regions is usually of high quality. Regions such as the Oqlid County, Shahr-e Kord and Torbat-e Heydarieh have the highest production of sugar beet in Iran. Sugar beet is widely adaptable to different environmental conditions. It is relatively resistant to coldness and heat and tolerates salty soil as well. This is a biennial and dicotyledon plant, which is economically important due to its unique potential for production of large amount of sugar (SALEHI *et al.*, 2006). Sugar beet is a diploid with 9 chromosome pairs and 9 basic chromosomes ( $2n=2x=18$ ).

Different plant genetic materials are considered to be potential reserves and precious supports by plant breeding experts. Although human has been one of the causes of the reduction in the genetic diversity of plant species, plant breeding experts have launched increasing and continuous measures to collect, maintain and study these genetic resources. To effectively devise breeding programs, information on the genetic diversity (GD) and relationships between sugar beet accessions is essential to allow selection of parents with desirable traits (IZZATULLAYEVA *et al.*, 2014). GD in sugar beets and related species and subspecies has been examined at a number of levels. For instance, quantitative root traits were used to evaluate GD in sugar beet populations from 3 different breeding programs (CURCIC *et al.*, 2013).

Plant genetic resources not only play an infrastructural role in the production of new cultivars and development of cultivation area, but also act as useful source of resistance to live and dead stresses as well as development of genetic compatibility with environmental changes (AZIZI *et al.*, 2001). Producing new superior cultivars is one of the important goals of breeders. Crossing new cultivars and selection superior genotypes for favorite traits in their progenies is one of frequently used methods (GOLPARVAR, 2013).

Genetic variation is necessary for successful breeding. In a breeding programme it is therefore of utmost interest to quantify the variation among breeding lines and wild relatives of the crop. Until recently, the predominant method for studying genetic variation has been isozyme analysis (GOLPARVAR and GHASEMI PIRBALOUTI, 2010). In recent years, the methods for detection and evaluation of genetic diversity have ranged from analysis at the morphological trait level to biochemical and molecular investigation. Molecular markers in particular are valuable for the rapid and reliable characterization of genetic diversity and are an important prerequisite for the efficient protection and conservation of genetic resources and their targeted exploitation in plant breeding. Currently, at least 15 types of DNA markers are used for molecular genetic analysis of plant genomes. Popular molecular markers for diversity analysis include restriction fragment length polymorphism (RFLP), cleaved amplified polymorphic sequence (CAPS), sequence-tagged

site (STS), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), sequenced characterized amplified region (SCAR), amplified fragment length polymorphism (AFLP), and intersimple sequence repeat (ISSR) markers (KHLESTKINA and SALINA, 2006; BABAYEVA *et al.*, 2009; ATES SONMEZOGLU *et al.*, 2012).

RAPD marker have a number of advantages for use in the detection of genetic variation such as technical simplicity, rapidity of assay, minimal DNA requirements, and low assay cost. In addition, no prior knowledge about the sequence under investigation is required. RAPD marker has been used successfully in diversity analysis of a number of crops (ALIYEV *et al.*, 2007; SURGUN *et al.*, 2012).

Three general criteria have been proposed for varietal identification; distinguishable intervarietal variation, minimal intravarietal variation, and environmental stability and experimental reproducibility. The third criterion has now been shown to apply to characters derived from RAPD for a range of plant material (GOLPARVAR and HEJAZI DEHAGHANI, 2012).

Accordingly to the above discussion and due to the economic and agricultural significance of sugar beet as well as the necessity of understanding the genetic diversity of different genotypes of sugar beet a number of thirty sugar beet genotypes were assessed using RAPD marker, which is suitable for the analysis of the genetic diversity of genotypes of sugar beet. In addition, knowledge of the genetic diversity of the genotypes of sugar beet is useful for breeding programs and identification/application of more suitable genotypes.

## MATERIALS AND METHODS

### *Plants in Use*

This experiment was carried out in the Plant Research Department at the Agricultural Research and Natural Resources Center of Isfahan Province. This department is located at the eastern longitude of 51°36" and northern latitude of 51°34". Its altitude is also 1500 m. Treatments include thirty genotypes of sugar beet. Specifications of the plant materials used in this study are shown in Table 1.

For the purpose of this research, transparent talcose vases with dimensions of 15\*4\*4 cm, sterilized using bleach. The vases were sterilized and filled with sand (with grading 11). The information about each genotype was also recorded on the relevant labels. Each vase contained one bush. Seedlings were irrigated using the Hoagland nutrient solution every seven days. Zarbar liquid fertilizer (with concentration of 2×1000) was also sprayed on leaves.

### *Laboratory Operations*

#### *Harvesting and Weighing Samples*

The harvest operation was performed 45 days after the planting the cultivars in a greenhouse in order to determine the genetic diversity of the cultivars. The harvested young leaves were put into a nylon with labels referring to the specifications of the leaves. Next, the nylons were transferred to the laboratory for DNA extraction purposes. Since the RAPD analysis method is highly sensitive and purity of DNA influences the accuracy, precision and repeatability of the results, in order to extract DNA 0.2 grams of fresh young leaves was first separated from each cultivar using a scale (with precision of 0.001). Afterwards, samples were transferred to Cetron micro tips with diameter of 2 mm using powder liquid nitrogen. The micro tips were subjected to a temperature of -20 °C (in a freezer) to be prepared for the next phase.

*Table 1. List of specifications of sugar beet genotypes under study*

Number	Genotype Number	Origin
1		SB27-HSF-2
2		SB31-HSF-1
3		SB31-HSF-5
4		SB31-HSF-7
5		SB31-HSF-9
6		SB32-HSF-1
7		SB32-HSF-3
8		SB32-HSF-5
9		SB32-HSF-9
10		SB33-HSF-1
11		SB35-HSF-4
12	31967	261*(20314*W-1009)-F2-S1-11-S1-3
13	31970	16261*(20314*W-1009)-F2-S1-11-S16
14	31976	(7112*SB36)*S1-3
15	31982	(7112*SB36)*S1-16
16	32003	SB31
17	32005	SB33
18	32008	(7112*SB36)*SB31
19	32166	S1_89074
20	32168	SB28_HSF_2
21	32170	SB34_HSF_1
22	32172	SB34_HSF_5
23	32174	SB34_HSF_13
24	32178	NE-0910-HSF-21
25	32180	NE-0910-HSF-43
26		SB-1
27		SB-3
28	F-20656	F-20656
29	F-20710	F-20710
30	F-20747	F-20747

*DNA Extraction*

There are different methods of extracting DNA from plants. Most of these methods take the following two issues into account: 1) quality of the extracted DNA; 2) extraction speed. In this research, DNA extraction was carried out using the C-TAB (GANESH, 2007) method.

*Polymerase Chain Reaction (PCR)*

The Polymerase Chain Reaction (PCR) was carried out using a thermal cycler with capacity of 25 microliter with the following elements: 1 microliter of genomic DNA with concentration of 50-60 Ng; 2.5 microliter of PCR buffer 10X; 1 microliter of dNTPs; 2 microliter of random primers with concentration of 10 PM; 0.3 units of taq polymerase enzyme; 2 microliter of 15 mgcl<sub>2</sub>; and 16.2 microliter of twice distilled water.

The thermal cycle included one phase of primary denaturing at a temperature of 94 °C for 5 minutes and 45 thermal cycles. In each cycle, the duration and temperature of denaturing were 1 minute and 94 °C, respectively. Moreover, the duration and string expansion temperature were also 1 minute and 72 °C, respectively. The primer connection stage took 1 minute at a temperature of 37 °C while the final expansion stage took 7 minutes at a temperature of 72 °C.

All PCR products were subjected to electrophoresis along with DNA molecular markers on 2% Agarose gel with constant voltage of 70 volts and equal conditions. After coloring gels by ethidium bromide and decolorizing gels with distilled water, images of gels were taken using Gel Document under ultraviolet light.

#### *Test Primers*

The random primers included OPA-09, OPA-10, OPA-19, OPB-18, OPC-06, OPD-03, OPE-01, OPE-12, OPP-17 and OPP-18 with sequences shown in Table 2.

*Table 2. Nucleotide sequence of primers in use*

Number	Primer name	Nucleotide sequence	Tm
1	OPA-09	GGGTAACGCC	34
2	OPA-10	GTGATCGCAG	32
3	OPA-19	CAAACGTCGG	32
4	OPB-18	CCACAGCAGT	32
5	OPC-06	GAACGGACTC	32
6	OPD-03	GTCGCCGTCA	34
7	OPE-01	CCCAAGGTCC	34
8	OPE-12	TTATCGCCCC	32
9	OPP-17	TGACCCGCCT	34
10	OPP-18	GGCTTGGCCT	34

#### *Statistical Analysis*

Statistical calculations were performed on the basis of the Jaccard similarity coefficient and UPGMA-based grouping in the NTSYS software (version 2.02). To this end, presence of a band was defined as 1 and absence of the band was defined as 0. The Jaccard similarity coefficient for random primers in the test genotypes of sugar beet was calculated. In addition, sugar beet genotypes were clustered using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The Cophenetic coefficient for the similarity matrix and the resulting curve was calculated as well (JUSTESEN *et al.*, 2003; CARLING *et al.*, 2000). For the purpose of statistical analysis, identical bands were omitted and only polymorphic bands were used. DNA multiplied segments varied between 100 and 3000 bp. For the accurate differentiation of genotypes and estimation of the similarities between the genotypes, adequate precision was employed in rating and omission of artificial bands. The artificial bands were bands that remained as bands with variations of PCR conditions and gel obscurity. The reason was that lack of precision in rating and presence of artificial bands leads to inaccurate estimation of genetic relationships between individuals. The

resulting binary matrix, which was used to study the diversity of genotypes, included 30 rows and 96 columns. The rows contained genotypes and columns contained polymorphic bands.

#### RESULTS AND DISCUSSION

Of the 40 random OP primers under study, 10 primers caused polymorphism and others either produced no band or produced multiplied products lacking clarity. Among the primers used in this research, the OPB-18 primer produced the highest number of bands (12 bands) and the OPA-09 primers produced the lowest number of bands (5 bands). The number of segments multiplied by different primers varied and the number of bands produced by random primers varied between 5 and 1 bands. The average number of bands produced by each primer was 9 bands (Figures 1-3). The OPA-10, OPC-06 and OPP-18 primers produced the highest number of polymorphic segments. The polymorphism of the random primers also varied from 100 to 57.14%. The OPD-03 and OPA-09 caused the lowest (57.14%) and highest (100%) levels of polymorphism, respectively. The average polymorphism for the random primers was determined to be 82.33%. In this study, a Cophenetic correlation coefficient of  $r=0.75$  was obtained from the dendrogram and similarity matrix. This value reflects the similarity matrix fits with the dendrogram satisfactorily. A Cophenetic correlation coefficient equal to/larger than 0.9, between 0.9 and 0.8, between 0.8 and 0.7, and less than 0.7 shows excellent, very good, good and poor fittings, respectively. The highest similarity was observed between cultivars 4 and 18 with a similarity coefficient of 91% while the lowest similarity was observed between cultivars 21 and 18 with a similarity coefficient of 0.63%. Cluster analysis also confirmed the results of genetic differentiation of the test cultivars as well as the correlations obtained from the Jaccard similarity coefficient. Results obtained from random primers helped classify different genotypes of sugar beet into thirteen groups.

Accordingly, the first group included cultivars 1, 16, 17, 19, 4, 18, 5, 26, 12, 6, 7, and 8 where cultivars 4 and 18 had the highest similarity with a similarity coefficient of 0.91. The second group included cultivars 11 and 14 with a similarity coefficient of 0.86. The third group only included cultivar 20 with a similarity coefficient of 0.82. The fourth group included cultivars 10, 22, 23 and 24 where cultivars 23 and 22 had the highest similarity (with a similarity coefficient of 0.88) and cultivars 10 and 24 had the lowest similarity (with a similarity coefficient of 0.80). The fifth group included cultivars 25 and 27 with a similarity coefficient of 0.83. The sixth, seventh, eighth, ninth, and tenth group included cultivars 9, 13, 2, 3 and 15, respectively. The similarity coefficient for each of the latter groups was 0.82. The eleventh group included cultivars 29 and 30 with a similarity coefficient of 0.85 and the twelfth group included cultivar 28 with a similarity coefficient of 0.82. Furthermore, the thirteenth group included cultivar 21 with a similarity coefficient of 0.8. Cultivars 15 and 21 with a similarity coefficient of 0.62 had the lowest similarity compared to all cultivars. In sum, the following factors influence the estimation of genetic relationships between individuals: the number of markers in use; distribution of markers in the genome; and nature of evolutionary mechanism (which form the basis of genetic diversity). According to the results of the present study it can be concluded that sugar beet genotypes from the same group reflect the kinship relationships between these genotypes and other genotypes. In addition, genotypes from the same group are closely related. Therefore, they are either originated from a common population or they had been originally one genotype named differently over time due to unknown reasons.

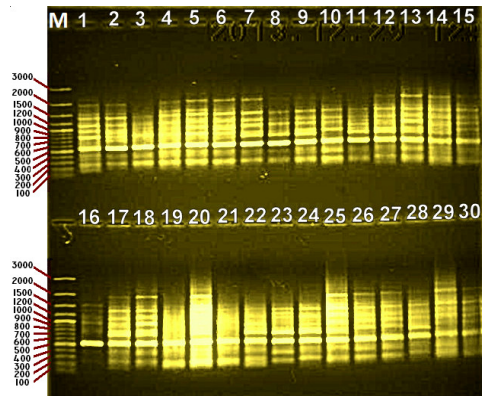


Figure 1. The band pattern of OPA-10 random primer

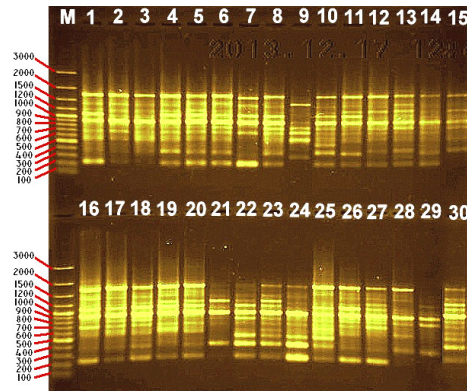


Figure 2. The band pattern of OPB-18 random primer

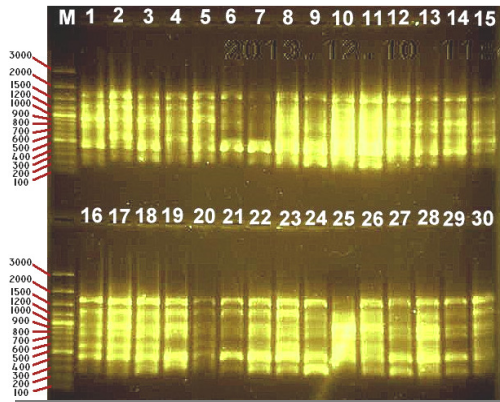


Figure 3. The band pattern of OPA-19 random primer

RAHMANI *et al.* (2012) used the following RAPD random primers: OP-D-13, OP-G-02, OP-H-03, OP-AA-09, OP-L-10, OP-K-20, OP-H-15, and OP-AH-09. The OP-D-13 and OP-G-02 primers produced bands with lengths of 1300 bp and 850 bp in the resistant bulk while such bands were not observed in the sensitive bulk. The diversity and difference in the banding pattern caused by the OP-G-02 primer was not seen in plants and polymorphism was observed. However, in the case of OP-D-13 primer, no difference was made to the banding pattern.

SRIVASTAVA *et al.* (2007) used two PCR-based molecular markers (RAPD and AFLP) to assess the genetic relationships and genetic diversity within/between five sugar beet genotypes. In sum, 383 band of 420 bands produced by 32 RAPD primers demonstrated polymorphism while the 5 AFLP primers produced 341 bands, 275 of which demonstrated polymorphism. On average, each primer produced 55 polymorphic bands. Results indicated that the degree of polymorphism caused by the AFLP marker was smaller than the RAPD marker.

In the other study, the RAPD primers generated 204 amplification products and the ISSR primers produced 178 fragments, 190 and 173 of which were polymorphic, respectively. The average polymorphism level with the ISSR markers (97.2%) was higher than that with the RAPD primers (93%). High genetic diversity indices for both marker types (0.86 for RAPD and 0.91 for ISSR) suggested that these methods were equally effective in determining genetic variation in sugar beet accessions (IZZATULLAYEVA *et al.*, 2014). Cluster analysis of the RAPD, ISSR, and combined datasets revealed similar grouping patterns. However, the dendrogram created from analysis of the combined RAPD+ISSR data was more similar to the RAPD-only dendrogram than the ISSR-only analysis, indicating that RAPD could determine genetic diversity with higher resolution than ISSR in the cultivars tested. High correlation between the RAPD and ISSR marker systems was shown using a Mantel test ( $r = 0.92$ ). Screening a higher number of anonymous loci in sugar beet using these molecular markers will enable the selection of the best parent cultivars for the development of novel varieties (IZZATULLAYEVA *et al.*, 2014).

SMULDERS *et al.* (2010) carried out a study to compare the characteristics of varieties of sugar beet using microsatellite markers. In this research, 25 new microsatellite markers of sugar beet were identified. A total of 12 markers with high qualitative patterns are used to describe 40 diploid and triploid varieties. As a result, 30 plants were subjected to molecular assessment for each variety. Markers multiplied 3-21 different alleles. Varieties partly showed 7 different alleles in one locus for each marker. The genetic differentiation among diploid varieties was shown to be constant based on the markers. In addition, the genetic differentiation among triploid varieties was much lower. In this research it was concluded that all varieties can be identified using 12 known markers. Markers can also be used in mapping and molecular breeding. They can also be used in studies of gene flow of crops to wild populations.

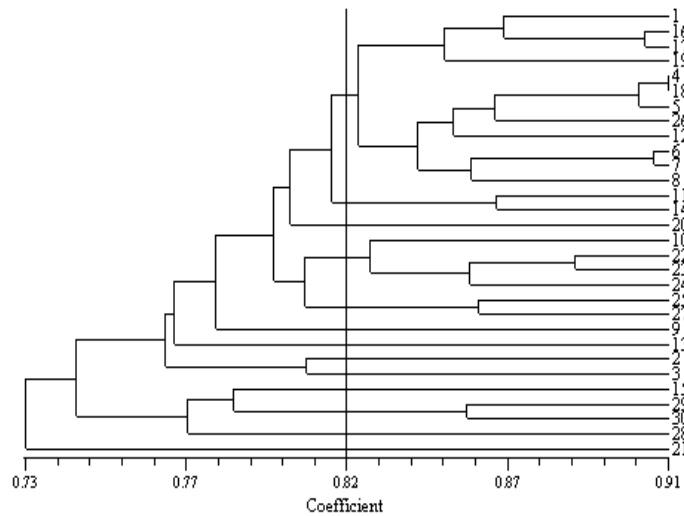


Figure 4. Dendrogram of cluster analysis of genotypes



## CONCLUSION

Molecular methods assess genetic diversity at DNA level or based on the origin of all plant characteristics. Hence, grouping based on the results of these methods is more useful than cluster analysis performed on morphologic data. The reason is that errors made in the course of measuring morphologic traits are more than errors made by laboratory methods. Therefore, environmental impacts may also be involved in the estimation of genetic diversity. In addition, the time required for analyzing genetic diversity on the basis of RAPD markers is much higher than the time required for measuring morphologic traits. Results of this research revealed that common methods can be used for identifying genetic diversity among different genotypes of a specific species (sugar beet genotypes in the case of this research). However, in order to complete and confirm the results, the RAPD method can be used to identify the associated cultivars. This way the genetic structure and diversity of cultivars can be identified in a short period of time.

Finally, according to the resulting dendrograms, there are probably genetic similarities between the cultivars of sugar beet and they probably have one common origin. Moreover, it seems that genotypes with less kinship relationships are more useful for breeding programs.

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### ANALIZA GENETIČKE DIVERGENTNOSTI GENOTIPOVA ŠEĆERNE REPE PRIMENOM METODA RAPD

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#### Izvod

Vršena su ispitivanja genetičke divergentnosti 30 genotipova šećerne repe korišćenjem metoda RAPD (Random Amplified Polymorphic DNA marker) Od 40 korišćenih prajmera. 10 su polimorfni i daju dobre jasne trake. Statistička analiza je vršena na principu koeficijenta sličnosti (*Jaccard*), a grupisanje *UPGMA* metodom u *NTSYS* programu (verzija 2.02). Utvrđen je polimorfizam svih prajmera (82.33%) unutar limita sličnosti. Koeficijent za matriks sličnosti i rezultirajuća kriva je imala  $r = 0.75$ . Analiza dendograma je potvrdila rezultate dobijene iz profila koje je dala genetička diferencijacija genotipova kao i korelacija koje su rezultirale iz koeficijenta sličnosti. Genotipovi su na osnovu dendograma grupisani u 13 grupa. Dobijeni rezultati su potvrdili da je genetička divergentnost među genotipovima rezultat efikasne selekcije.

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