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Evaluation of Genetic diversity of sugarcane variants using morphological characters and RAPD molecular markers.

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ABSTRACT

This study was accomplished to analyze the genetic variation of $r\circ$ agricultural sugarcanes variants using RAPD technique. The aim was to determine the genetic variation level and relation among some commercial varieties of *Saccharum officinarum* and the two wild varieties of *Saccharum spontneum* as well. Total genomic DNA of each sample was extracted from $1, \circ, \tau, \circ$ gr of new germinated fresh young leaves using modified CTAB method. The concentration was determined by spectrophotometer. RAPD analysis was performed by use of $1 \cdot$ bp randomized nucleotide primers. All genotypes were studied for presence or absence of bands, and analyzed by use of statistical software NTSYS $1, \tau, \tau$. Among $1\circ$ used primers, r.

of them showed polymorphism between the sample, and $\xi \tau \tau$ polymorphic bands were produced. Cluster analysis lays varieties into two separated groups through the Jacard coefficient and UPGMA methods. In this classification, the two wild varieties from the *Saccharum spontanenm* were separated completely and showed completely different band pattern in comparison with other varieties. Generally, the results of this research showed that RAPD marker is a suitable tool to study genetic variation in sugarcane. It is recommended to increase the efficiency of breeding programs to study precise genetic variation and to determine the relationship among cultivars. Overall, the results indicated that RAPD technique in combination with morphological selection and identification could serve as an ideal tool for studying the genetic variation among sugarcane families.

Key words: Sugarcane, RAPD molecular marker, Genetic variation, morphological characteristics.

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INTRODUCTION

Sugarcane belongs to the grain (*Gramineae*) family and *Saccharum* genus [1, 7]. In this genus there are three agricultural species of *Saccharum Officinarum*, *Saccharum Barberi* and *Saccharum Sinense* and two wild species of *Saccharum Spontaneum* and *Saccharum Robustum*. Although these species differ in the chromosome number, cross-breeding is possible within the genus [7].

Sugarcane is considered one of the most important sugar crops worldwide. This crop can produce high quality sugar and in large amount per unit area of land [1, "]. Beside sugar, leafs, branches, Bagasse, molasses and chemical products are among sugarcane by-products that have economic value [1]. Because the germplasm modification and breeding is an active process in sugarcane production, study of genetic variation and determination of relative genetic distance between individuals or populations of sugarcane is an important aspect of breeding programs. Furthermore, knowledge in genetic similarities among different genotypes in complement with phenotype information will significantly aid germline modification programs.

¹ Considering that genetic analysis of seed sources and seed collections in an organizes and classified manner in an expensive and time-consuming approach, use of inexpensive and simple molecular markers such as Random Amplified Polymorphism DNA (RAPD) could

simplify the process of genotyping and acquiring necessary information regarding the source of crops [[¢]]. In this method specific sequences within the genome are targeted and amplified using ten nucleotide-long primers in a PCR reaction [°].

Despite the dominance of the low repeatability of the RAPD technique, this method is used to assess the genetic diversity among plant species and populations [1]. Leon et al. [V] used RAPD technique and identified high genetic diversity among rq species of sugarcane and used the data for breeding programs and selection of suitable parents.¹ Nair et al. [$^{\circ}$] used RAPD technique for identification and genetic variation analysis of Indian sugarcanes and recognized specific band patterns for different variants. Karp et al. [$^{\wedge}$] used RAPD technique for evaluation of difference in various genotypes and germplasms in *Saccharum* genus and reported poor general variation among the genus. The reason of low genetic diversity among Indian sugarcanes could be explained by repeated used of few parent sugarcanes in the breeding programs [q].

Considering the importance of sugarcanes in sugar production and agriculture industry of northern and southern of Iran, identification of available genotypes for breeding programs and development of more productive crops is necessary. Identification of productive crops with high yield for cultivation in south regions of Iran enables the breeding program to limit the financial resources to focus on producing crops with the highest yield and modify the genotype for the best quality and quantity product. Identification of high yield crops with similar genotypes and recognizing the genetic diversity among variants results in persistent production and spread of crop with the highest yield in much shorter time.

The purpose of this study is to classify the genotype of the sugarcane crops in Iran and obtain a relationship among different genotypes, and also to study the similarities and differences in the genetic materials of these crops. Such data can be valuable in the breeding programs of the sugarcane crop.

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MATERIALS AND METHODS

Sampling: The genetic material used in this research was consisted of $\uparrow \circ$ samples from $\uparrow \cdot \cdot$ domesticated *Saccharum Officinarum* sugarcanes and \uparrow samples of wild type *Saccharum Spontaneum* and \uparrow *Saccharum hybrid* samples imported to Iran from various sources. This selection was chosen from the earliest and the most recent sugarcanes archived by the **Ahvaz**

Sugarcane Research and Development Center and was selected by the center and included in this study based on yield, variety, adaptation to the region and relationship.

All sample leafs were transferred from Amirkabir Sugarcane Development Center in Ahvaz on ice to Biotechnology Laboratory of Shahrekorde University. After removal of extensions and main veins, leafs were weighted and kept in liquid nitrogen until further application.

DNA extraction: DNA was extracted from $1, \circ- \mathfrak{r}$ g of leafs using modified CTAB method $[1 \cdot]$. DNA was dissolved in Tris-EDTA (TE) buffer and quantified using Eppendorf biophotometer.^{\tilde{l}} The samples were diluted in a ratio of $\mathfrak{r}:\mathfrak{h}$ in TE buffer and read at \mathfrak{r} . m. The below formula was used to calculate the amount of DNA in each sample.

 $A^{\gamma\gamma}A^{\gamma}A^{\gamma}$ ratio was used to determine the DNA purity. Samples resulted in higher value than γ , were discarded and DNA extraction was repeated. DNA quality was analyzed using Agarose gel electrophoresis [γ].

RAPD technique: Total of $\uparrow \cdot$ primers were synthesis by MWG Biotech Company (Germany) and used for initial evaluation. From which, $\uparrow \cdot$ primers that resulted in constant and sharp bands were selected. The PCR reaction was carried out according to the method described by Williams et al. [$\uparrow \uparrow$] with minor changes in a $\uparrow \circ \uparrow \mu$ l reaction. The reaction mixture was consisted of $\uparrow , \circ \dagger \mu$ l ($\uparrow \cdot X$) PCR buffer, $\uparrow \cdot \cdot mM$ dNTP mixture, $\uparrow \circ$ ng of each primer, $\cdot , \uparrow v$ units of Taq DNA polymerase and $\circ \cdot$ ng of template DNA.

After $^{\text{m}}$ min of initial denaturation at $^{97^{\circ}}$ C, total of $^{\xi\xi}$ cycles of; 1 min at $^{97^{\circ}}$ C, 1 min at $^{70^{\circ}}$ C and 7 min at $^{97^{\circ}}$ C were carried out and followed by final extension of 1 min at $^{97^{\circ}}$ C [17].

Presence or lack of each specific band was scored \circ or \cdot respectively. Data was uniformed using SEQUID analytical software. Weak or un-repeatable bands were ignored. A matrix file was designed, the columns represented genotypes and each rows correspondent to a specific band. A distance matrix and similarity matrix were designed and calculated based on Jaccard similarity coefficient. Classification was carried out by UPGMA method using NTSYS software ($\gamma \cdot \gamma \gamma$ Version).

One method to compare the efficiency of different clustering algorithms, is estimation of cophenetic coefficient correlation, goodness of fit criterion for grouping [ξ] is the correlation matrix of similarity or distance (Y), by cophenetic matrix (X) by using the options of COPH and MAXCOMP in NTSYS software. The method which shows the highest Cophenetic

coefficient correlation may be considered as the most appropriate method of analysis and could be applicable.

Measurement of quality factors of sugarcanes: The quality factors of each sample were measured by observation in a farm. Scores of 1 to 1, were given to quantify the morphological characteristics (Table 1).

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RESULTS

Total of $\[\] of \[\] primers were used in this study and \[\] of those resulted in repeatable multi-band$ $amplicons with specific bands size (Figures)). The primers resulted in <math>\[\] AA$ bands with specific sizes. Each specimen showed different band pattern which indicates the genetic diversity among this samples. Band were in a size range of $\[\] AYY \] bp$ to $\[\] AYY \] bp$. Majority of primers resulted in a distinctive band pattern in wild *Saccharum Spontaneum* compared to all $\[\] YY \] variants of$ *Saccharum Officinarum*. Including the two wild stains of*Saccharum Spontaneum* $; Spont \] and Spont \], the number of different bands with distinguish patterns were$ $<math>\[\] YY \] which indicated the polymorphism of <math>\[\] YY, \] AX$. The variety of bands in a *Saccharum Officinarum Officinarum* specie was $\[\] Y, \] YY \] and included \] YY \] distinct bands (Table Y).$

This result conforms that this technique can be used to classify various genus and species and is a reliable method for using in a breeding programs and genetic reformation of sugarcane species.

Cluster analysis dendrogram divided the samples into two main group with distance of $\forall \forall \ddot{\chi}$ (Figure \forall). In this cluster $\forall \circ$ samples were sorted into two mail groups.

First group was divided in ten subgroups which included PINDAR, NI⁴, NCo^r) · and B^{ϵ}^r · variants. NI⁴ strain was imported from Japan and NCo^r) · was imported from Coimbatore, India had a genetic distance of ^r · ^r and were sorted in one group. Maternal parent of NI⁴ strain was NCo^r) · [^r]. Genetic distance of PINDAR variant originated from Australia was ^r · ^r from NI⁴ variant. With the exception of B^{ϵ} · ^r · ^r variant, all variants of this group had a common ancestor.

The second subgroup comprised from, VESTA and TRITON variants from Australia, TUC¹, variant from Argentina, and C¹, from Cuba. In this cluster VESTA and TRITON

variant had a genetic distance of 19% and TUC1A-19 and CA1-201 had a genetic distance of 12%.

The third subgroup contained the valuest of SP^{1-117} , SP^{1-1117} and RB^{7-0019} from Brazil. The genetic distance of SP^{1117} and SP^{1-1117} was calculated ¹⁰%.

The CSG^{Λ , 77°} and CSG^{Λ , 7° Λ} variants originated from Cuba with the genetic distance of $^{\Lambda}$, were assigned to the fourth group.

The fifth cluster included variants $My^{\circ\circ-1} t C^{\circ} t C^{\circ} t$ and $JA^{\circ} t t$ from Cuba and $N^{\circ} t$ variant from South Africa. The genetic distance of $Ja^{\circ} t t$ and $C^{\circ} t t t$ was $t^{\circ} t$ and the $Ja^{\circ} t t$ and $My^{\circ\circ-1} t$ was calculated $t^{\circ} t$. The genetic distance of $N^{\circ} t$ variant and $Ja^{\circ} t t$ was $t^{\circ} t$.

The sixth subgroup contained '• strains from Louisiana and Florida, USA and CRISTALINA variant from Guinea. The CRISTALINA variant is a natural hybrid from the *Saccharum Officinarum* genus and is ancestor of these '• strains.

The seventh subgroup included the two variants of $COLK^{\land, \circ}$ and CO°_{\land} from Coimbatore, India. The $COLK^{\land, \circ}$ is a maternal parent of CO°_{\land} .

MEX° γ - $\xi \gamma \gamma$ and COK $\gamma \gamma$ were clustered under the eighth subgroup and SP γ - $\gamma \xi \gamma$ was clustered under the ninth subgroup.

In the second group the wild type *Saccharum Spontaneum* was separated from the other agricultural strains and had a genetic distance of $\circ \gamma$? (Figure γ).

The clustering of the obtained data from this research divided the wild-type *Saccharum Spontaneum* from *Saccharum Officinarum* genus. These results are similar to the results obtained by Glaszmann [1°] and Schenck [1[°]] who used different molecular markers and is completely in compliance with the RAPD results. Furthermore, our results confirmed the results obtained by Selvi et al [1[°]], Alwala et al [1[°]] and Karami [1[^]] who used SSR and TRAP molecular markers respectively to classify the genus and species of Saccharum in separate dendrogram.

In the morphological classification, the dendrogram plotted based on quality traits divided the samples into four groups (Figure r).

The first group is divided into two subgroups. The first subgroup included; $CP^{\vee\gamma-\gamma} \cdot \Lambda^{\gamma}$, $CO^{\vee\gamma+\Lambda}$, CRISTALINA, $CL^{\vee\gamma-\gamma\gamma\gamma}$ and $B^{\xi\gamma-\gamma\gamma}$. The second subgroup included three variants of Spont[\], Spont[\] and MEX^{$\circ\gamma-\xi\gamma\gamma$}. All species in the first groups have raised leafs. In contrast to the second subgroup, the first subgroup had less adherent sheaths to the stem. Leafs in the first subgroup were all green or light green and the second subgroup variants had dark-green leafs. Unlike the second group, in the first group all stem had waxy texture. All members of group one had a cylindrical internodes. None of the second group members had corky leave, whilst, CRISTALINA, $CP^{\vee\gamma-\gamma} \cdot \Lambda^{\gamma}$ and $CP^{\xi\Lambda-\gamma} \cdot \gamma^{\circ}$ variant from the first group had corky leave.

The first group is separated from the second group with a distance of ξ , \cdot . The second group is divided into tree subgroups, the first and the second of those are separated at the distance of γ , $\cdot \gamma$, and the second subgroup is separated from the third subgroup at the distance of γ , $\cdot \Lambda$.

The second group comprised from; NCO^{π})·, NI^{η} TRITON, CP^{ν}·-^{π}^{τ}), CP^{ν}^{π}-^{τ}), L^{τ}^{τ}-^{τ}^{η}, N^{π}·, JA^{τ}^{ϵ}-^{τ}·, COLK^{\wedge}··^{τ}), RB^{\wedge}^{π}-^{\circ}°^{τ}^{η}. Members of this group have raised and semi-raised leafs and the adherent of the sheath to the stem is intermediate to strong. Leafs are dominantly green, except for NCO^{π})·, CP^{ν}^{π}-^{τ}), and CP^{ν}·-^{π}^{τ}) who had brown lines within a green background. Most stem contained cylindrical internodes. In the second subgroup of the second group, only the NI^{η}, TRITON, and L^{τ}/^{τ}-^{τ}^{η} variants had corky leaves. The leaf color is dominantly green, however some variants had green to yellow features. The second group is divided from the tired one at the distance of ϵ .^{τ}/^{τ}.

The third group comprised from $CSG^{\Lambda,-\sigma\circ\Lambda}$, VESTA, $SP^{\vee,-1}$, $CP^{\circ\vee,-1}$ and $TUC^{\Lambda,-1}$. 1.4. Leafs of the third group are semi-raised or flat. The sheaths have strong adherence to the stem. Leafs are usually dark-green and in the two variants of $SP^{\vee,-1}$ and $CP^{\circ\vee,-1}$ stems have high waxy texture whereas in the $TUC^{\Lambda,-1}$, $CSG^{\Lambda,-\sigma\circ\Lambda}$ and VESTA the stems have less waxy texture. The internodes are cylindrical and in the $CSG^{\Lambda,-\sigma\circ\Lambda}$, $SP^{\vee,-1}$ and $CP^{\circ\vee,-1}$ the internodes are narrower. The members of this group lack corky leaves and stem are dominantly green. The fourth group is separated from the third group at the distance of $^{\circ,\gamma\gamma}$. The fourth group includes three subgroups, and the first subgroup has distance of $^{\vee,\vee\gamma}$ from the second subgroup which itself is separated from the third subgroup at distance of $^{\vee,\vee\circ\xi}$.

The CP1^r- \circ AA, CSGAA-^r 1° , PINDAR, MY $^{\circ}$ - 1^{\sharp} , SP 1 - 111^{r} , CP1 1 , CP1 1 and CP 1 - 1^{4} variant are classified into the fourth group. These variants have raised leafs. The

adherence of sheath is weak to intermediate. Leafs are dark-green and three variants of SP^{γ})- $\gamma\gamma\gamma$, PINDAR and SP^{γ})- $\gamma\gamma\gamma$ have opaque dark-green leafs. In this group only MY^{\circ}- γ </sub> has light-green leafs. The stem of this variant has less waxy texture. In CP $\gamma\gamma$ - $\gamma\Lambda$ and MY^{\circ}- γ the internodes are barreled-shaped which is unique among all members. CP $\gamma\gamma$ - $\gamma\Lambda$ and MY^{\circ}- γ have corky leave. In all members except CP $\gamma\gamma$ - $\gamma\Lambda$ the stem has red color.

Generally in the dendrogram plotted based on the quality of the traits, the two variants of TRITON and L^{γ}-^{γ} had highest similarly and two variants of SP^{γ}-^{γ} and CP^{$\circ\gamma$}-^{γ} had the lowest similarity rate.^{\tilde{J}}

The highest correlation between the quality traits belonged to the color of the leaves and stems texture and the lowest correlation belonged to the waxiness of the stem and the shape of leafs.

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DISCUSSION

The RAPD technique used in this research was able to completely distinct between the sugarcane genus and even within the different species used in the study the obtained band pattern was unique and distinguishable.

These results are consistent with the reports published by other researchers including Huckett et al $[1^{9}]$ and Barⁱnek et al $[7^{1}]$ who reported RAPD bands are inherited in sugarcane families and can be used as a molecular marker to distinguish different variants of the crops.

In a closer look at the obtained dendrogram we realize that in addition to the different clustering of agricultural and wild-type strains, the samples were also divided based on parents, origin and similar nomenclature. However, among this r° variant, some heterogeneous variants that had similar names or origin were included in different clusters. Schenck et al [r] in a study on domesticated and agricultural sugarcane variant which had

similar naming and was expected to have identical clustering, reported that variants were divided into different clustering groups. Additionally, the new developed spices have high polyploidy and it is expected to have significant chromosomal overlap, thus there are more annealing sites for RAPD primers. The classification of these species using RAPD will not be useful and result in various and unrepeatable bands, however, this technique can be useful for diversity studies.

The results of cluster analysis based on the traits quality did not provide any useful data regarding the functionality of the traits and only revealed the correlation among the quality traits.

In this research there was a suitable diversity in the statistics and with the use of genetic distance calculated for each group, proper parents can be identified for breeding programs.^{$\tilde{1}$} Since the strains with the highest genetic distance have higher heterosis potential, cross between parents with the highest genetic distance will have better yield in production of hybrid strains

The overall results of this study shows that RAPD technique is a proper tool for analysis of genetic diversity in sugarcanes and it is recommended that for better yields in the breeding program, the strains with the highest genetic distance to be used. Example of that will be the cress of NCO^r · strain with the MEX^{$ov_- \xi v r$} strain. If the selection of breeding parents in the breeding programs would be solely based of morphological characteristics, the results may not be desirable.

Before use of molecular markers for analysis genetic relationships in certain species, morphological characteristics had the most significant role. Recently biotechnological tools has significantly improved phylogenic studies and detection of relationship among families of certain genus. Use of molecular markers and morphological traits are complementary in the breeding programs and separate use of each method without considering the other one may not lead to the best results. There are various studies that used statistical analysis and reported correlation between a molecular marker and a specific trait in plants [$\gamma r_{-}\gamma \gamma$].

Our results indicated that morphological characteristics are useful in identification of genetic diversity in sugarcanes. However, RAPD technique uses molecular markers and signatures and was successful in identification of genetic diversity. The RAPD technique is a useful tool for obtaining genetic diversity among different species and even within a family of sugarcanes and can be used to manage the germplasm resources.

The clustering results based on RAPD technique and morphological traits show somewhat similarities. The differences could be explained by post translational modification and non-nuclear inheritance of some traits. Therefore, as shown in this study, the diversity of genetic material in different species such as sugarcanes can be measured by a simple and cost-effective molecular method such as RAPD and the results can be used with high accuracy.

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Table '; Traits analyzed in morphological study.

	of the Stem Color	corky of Shape of the Waxiness of Co			Color of	Color of the Adherence of Shape		
		leave	internodes	stem	the leaf	sheath	the leaf	
Yellow-Green Yellow-red	Yellow	Seas	emi- cylindrical cone	low	Green	low	Raised	
Green-yellow Green-Red	Green	1 cus	Narrowed Barreled-shape	Intermediate	Light- Green	Intermediate	Semi- raised	
Light-red Dark-red	Red	No	Curved	high	Dark-	high	Flat	
Combination o	f yellow, green and				Green			
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Table ^Y: Results of RAPD. Number of bands, polymorphism and the name of primer used are indicated

polymorphic Percentage of polymorphic	Number of	Total of bands	Name of
bands	bands		Primer
11,11	ź	٦	۲OPM
۱	١٨	١٨	٤OPM
۱	۲ ۲	۲۲	٥OPM
۱۰۰	١٩	١٩	^OPM
V T , V T	٨	۱۱	NOPM
۱	٩	٩	OPM° (
97,70	10	١٦	NOPM
1	١٨	١٨	١٧OPM
٩١,٦٦	11	۱۲	\^OPM
٩.,٩.	۱.	۱۱	٩OPM
۱	١٨	١٨	۲۰OPM
۱	۲ ۱	۲ ۱	Y \OPM
٩ ٤ , ٤ ٤	1 V	١٨	۲ ۲ OPM
97,70	10	١٦	۲۳OPM
٨٨, ٣٣	10	1 V	oOPM وم
۱	11	11	۲VOPM

	1 • •	10	10	۲٩OPM
	٨٦,٦٦	١٣	10	۳۲OPM
	1	۲ ۲	۲۷	۳۳OPM
	1	۲ ۲	* *	oPM°
	1	١٦	١٦	OPM۳۶
	1	١٦	١٦	۳۷ΟΡΜ
	٨٢,٢٣	10	1 V	۳۸OPM
	٨٤,٦١	11	١٣	٤ · OPM
	۱	1 V	١٧	٤٢OPM
	۱	۲.	۲.	٤٨OPM
	۱	4 4	* *	۰OPM
	۱	۲ ۱	۲ ۱	٥°OPM
	٦.	٦	۱.	٥٧ОРМ
	٩٣,٣٣	۱.	۱ ٤	۰OPM
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Figure 1: Electrophoresis results of amplified DNA using random primers,

From top to down, respectively; $\tilde{j}~OPM(\circ\circ).$

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Figure γ : Plotted dendrogram based on Jaccard coefficient for $\gamma \circ$ variants of sugarcane based on RAPD results.

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Figure ^{*}: Classification of sugarcanes based on quality of traits using Jaccard coefficient.