Molecular differentiation for six genotyps of sugarcane (*Saccharum* spp.) using scot marker

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ABSTRACT

Start codon targeted polymorphism (SCoT), a novel molecular marker targeting conserved region surrounding start codon proved its efficiency in genetic diversity analysis and fingerprinting. In this study, 151 amplified fragments were obtained by using 10 SCoT primers with 6 genotypes of sugarcane (Saccharum spp.), of which 98 (64.9%) were polymorphic. And, there were 44 unique fragments divided into positive and negative markers related to important characters in sugarcane species. Highest percentage of similarity (87.32%) was found between genotype 5 (G. 54-9) and genotype 6 (BOT 41) utilizing NTSYSpc 2.01b software through UPGMA to find the genetic relationship illustrated in phylogenetic tree.

Key words: *Genetic diversity, Sugarcane, SCoT molecular markers, Saccharum spp.*

INTRODUCTION

Sugarcane is the common name for a species of herb belonging to the grass family. The botanical classification of sugarcane is *Saccharum officinarum*, family: Poaceae. Sugarcane is a tropical crop requiring a hot climate. However, it also grows well in subtropical climate. The primary use for sugarcane is to process sugar, which is used in different products; food, fiber, fodder, Fuel and cemicals (Dotaniya *et al.,* 2016). In Egypt, sugarcane crop is considered as one of the important industrial crops and is the main source of sugar production. Also, it is used in Egypt for fresh juice consumption and molasses industry. Sugarcane breeding program places considerable emphasis on producing new high yielding varieties, resistant to diseases, insects and characterized with high rationing ability (Khaled and Ahmed 2008). The Egyptian germplasm contain local genotypes and genotypes imported from different breeding stations around the world. Genotypes G. 95-21, G.T. 54-9 and G. 99/103 were developed, evaluated under Egyptian conditions and released in Egypt (Khaled 2010). In addition; to developing new promising cultivars, agricultural practices must be improved for increasing sugarcane production (Fahmy *et al.,* 2008). Biotechnology has been used as a tool to increase agricultural productivity in the context of sustainable agriculture, especially the utilization of molecular markers (RFLP, RAPD, ISSRs and SSRs) for studying genetic diversity, cultivar identification and for marker-assisted selection (MAS) of major crops such as rice, maize, wheat and sugarcane (Fahmy *et al.,* 2008 and khaled, 2010).

Recently, many new promising alternative marker techniques have been

utilized. These techniques implicate inter-retrotransposon amplified polymorphism, retrotransposon microsatellite amplified polymorphism, sequence-related amplified polymorphism, and target region amplified polymorphism (Collard and Mackill, 2009). Coupled with the fast development of genomics research, there has been another direction away from random DNA markers towards gene-targeted markers. Also, data obtained from genome sequence offers enormous potential for the development of new markers and detect gene(s) in diverse plant species (Collard and Mackill, 2009).

In this study, we exploited a novel marker system, namely start codon targeted polymorphism (SCoT), based on the short-conserved region in plant genes surrounding the ATG translation start (or initiation) codon that has been well characterized in previous studies (Collard and Mackill, 2009). The technique is simple, low cost and highly polymorphic and provides extensive genetic information. Moreover, its primers are universal in plants. This marker system has been successfully employed in genetic diversity analysis and fingerprinting of many agricultural and horticultural crop species (Satya *et al.,* 2015). This newly reported method was successfully tested with sugarcane genes by Wu *et al.* (2013), due to its beneficial qualities in differential gene analysis in comparison to other methods. The aim of this study was to apply SCoT molecular technique as a novel method in the analysis of genetic diversity of sugarcane genotypes (*Saccharum* spp.) and the species (*Erianthus arundinaceus*).

MATERIALS AND METHODS

Plant materials and DNA extraction

Six genotypes of sugarcane (*Saccharum* spp.), and the species (*Erianthus arundinaceus*) (Table 1), were kindly obtained from Sugar Crops Research Institute (ARC), Giza, Egypt. Total DNA was extracted from newly developed leaves using Graham and Henry (1997) method, as it is a rapid DNA isolation method. The quality and quantity of DNA samples were examined on 1% agarose gel and determined its size using 100 bp DNA ladder (Solis BioDyne) as a marker.

Table (1): List of six sugarcane genotypes and pedigree with their characterization.

Number	Genotypes	Characterization	Origin /Pedigree
1	IK 76-79	Parent/ high sucrose	*Erianthus arundinaceus* (a wild relative of sugarcane)
2	G. 95-21	Check variety	Egypt Hybrid (Sp. 79-2278 X Sp. 80-1043)
3	G. 99/103	Commercial/ high sucrose/ disease resistant except smut	Egypt Hybrid (US. 74-3 X Cp. 76-1053)
4	SP 80-3280	Parent/ disease resistant	(*Saccharum* sp.)
5	G.T. 54-9	Commercial/ high sucrose	Giza-Taiwan Hybrid (NCO 310 X F 37-925)
6	BOT 41	Parent	*Saccharum officinarum* L.

SCoT-PCR amplification

Ten SCoT primers were utilized (Table 2) according to Collard and Mackill (2009) and Wu *et al.* (2013). Amplifications were carried out in 25 µl reaction mixtures containing 17.8 µl sterile distilled water, 5 µl of 5x PCR buffer (containing $MgCl_2$ and dNTPs), 1 µl of each primer (0.7 µM), 0.2 µl (5 U) of *Taq* polymerase and 1 µl (50 ng) template DNA. Perkin Elmer DNA thermal cycler was used with the following PCR program: an initial denaturation for 5 min at 95 ^{o}C, 35 thermal cycles (1 min at 95 ^{o}C, 1min at 50 ^{o}C and 1 min at 72 ^{o}C) and a final 5 min extension at 72 ^{o}C (Collard and Mackill, 2009 and Wu *et al.,* 2013).

Gel analysis and phylogenetic relationship

PCR products were run in agarose 1.5% and gel image was analyzed using the TotalLab TL120 to determine molecular size of the amplified fragments according to 100 bp DNA ladder. Each variable SCoT fragment was considered as a locus, every locus scored as present (1) or absent (0). For data analysis, only polymorphic, reproducible, and clear-cut fragments were considered. Phylogenetic relationships were estimated using NTSYSpc 2.01b software through the unweighted pair-group method using arithmetic averages; UPGMA (Rohlf, 2000).

Table (2): SCoT primers name, sequences and their GC%.

Primer Name	Sequence (5'-3')	GC%
ScoT 5	CAACAATGGCTACCACGA	50
ScoT 7	CAACAATGGCTACCACGG	56
ScoT 10	CAACAATGGCTACCAGCC	56
ScoT 18	ACCATGGCTACCACCGCC	67
ScoT 22	AACCATGGCTACCACCAC	56
ScoT 26	ACCATGGCTACCACCGTC	61
ScoT 31	CCATGGCTACCACCGCCT	67
ScoT 32	CCATGGCTACCACCGCAC	67
ScoT 34	ACCATGGCTACCACCGCA	61
ScoT 35	CATGGCTACCACCGGCCC	72

RESULTS AND DISCUSSION

Rapid and precise genotype identification is extremely important for germplasm characterization and the practical breeding of different species. Initiating a trend away from random DNA markers towards gene-targeted markers, Start Codon Targeted (SCoT) polymorphism (Collard and Mackill, 2009) was applied, based on the short-conserved region flanking the ATG start cdon in plant genes. A total of 151 amplicons were obtained from PCR using the ten SCoT primers with 64.9% of polymorphism (Table 3).

Fragments ranged in size between the smallest amplicon 166 bp with primer S-18 to the largest one 3112 bp with primer S-26. Additionally, there were 22 positive markers and 22 negative markers as a total for the ten

primers. The highest number of amplicon was 27 fragments with primer S-7, while the lowest number was 9 using primer S-5. Also, the highest polymorphism percentage was 88.89% for primer S-7, whereas the smallest percentage was 41.18% for primer S-26. Fig. (1) illustrates an example of SCoT-PCR amplicons obtained with the 6 genotypes of sugarcane.

Table (3): Result of SCoT-PCR reaction from the ten primers with 6 genotypes of sugarcane.

polymor-phic %	Unique marker	Polymorphic fragments	Monomorphic fragments	Total number of amplicons	Range of size by bp	Primer name
55.56%	+(1)	5	4	9	445-2784 bp	S 5
88.89%	+(2), -(7)	24	3	27	226-2641 bp	S 7
42.86%	+(3), -(2)	6	8	14	276-1555 bp	S 10
66.67%	+(2)	8	4	12	166-1228 bp	S 18
57.14%	+(2), -(2)	8	6	14	269-1445 bp	S 22
41.18%	+(3)	7	10	17	336-3112 bp	S 26
78.57%	+(3), -(1)	11	3	14	205-1481 bp	S 31
56.25%	+(2), -(2)	9	7	16	188-1568 bp	S 32
80%	+(3), -(4)	12	3	15	226-1006 bp	S 34
61.54%	+(1), -(4)	8	5	13	169-956 bp	S 35
64.9%	+(22), -(22)	98	53	151	166-3112 bp	Total

+ indicates positive marker's number of fragments
- indicates negative marker's number of fragments

Table (4): Pairwise similarity indices using Dice (Nei and Li) of the 6 sugarcane genotypes.

5	4	3	2	1	
				0.7814	2
			0.7788	0.7411	3
		0.7677	0.7870	0.7610	4
	0.7678	0.7980	0.8054	0.8286	5
0.8732	0.7788	0.7400	0.8165	0.8406	6

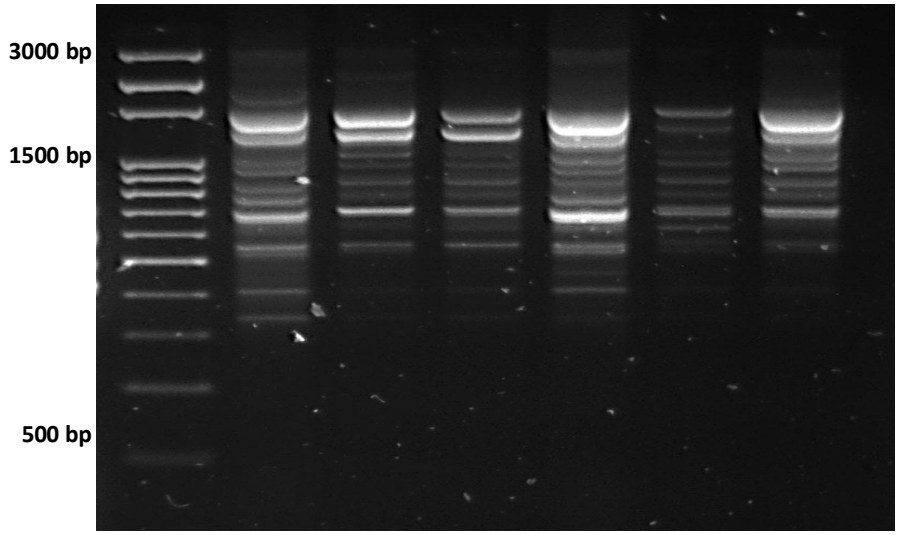

Fig. (1): SCoT-26 primer profiles as obtained with the 6 genotypes of sugarcane.

From the 22 positive markers, which is found by the ten SCoT primers, there were unique fragment that could be related to a special character in sugarcane. Five positive markers were found in genotype 1 (IK 76-79) that is used as a parent in breeding program; may be related to high sucrose content 1555 bp with primer S-10, 621 bp and 297 bp with primer S-18, 205 bp with primer S-31 and 384 bp with primer S-34. Also, there were five positive markers found in genotype 4 (SP 80-3280) that is used as a parent in breeding program; may be related to disease resistance at 620 bp and 455 bp with primer S-26, 214 bp and 188 bp with primer S-32 and 684 bp with primer S-34. The obtained fragments were scored as present (1) or absent (0) and analyzed using NTSYSpc 2.01b software through the unweighted pair-group method using arithmetic averages; UPGMA (Rohlf, 2000) to determine the genetic relationship (Table 4).

The highest percentage of similarity (87.32) was found between genotype 5 (G. 54-9) and genotype 6 (BOT 41). Fig. (2) demonstrates the phylogenetic relationships for the six genotypes of sugarcane and distinguished them into two main clusters, the first contains 99/103 genotype, while the second combined the other five genotypes which were in two subclusters; SP80-3280 in one and the other four genotypes in the second. The two genotypes (G. 54-9 and BOT 41) which were very close to each other as being *Saccharum officinarum* L. and related to IK 76-79 (*Erianthus arundinaceus*), then G. 95-21 was present with genotype SP 80-3280. As a conclusion, the obtained results: SCoT-PCR molecular markers proved their effectiveness, reproducibility and reliability in studying differentially expressed genes. SCoT markers are generally reproducible, dominant markers like random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) and could be used for genetic analysis, quantitative trait loci (QTL) mapping and bulk segregation analysis. So, SCoT-PCR molecular marker confirmed its ability to measure genetic diversity between spices as studied by Bhattacharyya *et al.,* (2013) to estimate the genetic diversity of *Dendrobium nobile* Lindl., and Paliwal *et al.,* (2013) who

investigated 21 accessions of Giloe (*Tinospora cordiolia* Willd. Miers ex Hook. F. and Thoms) which collected from two states of India using SCoT markers. In our study SCoT differentiate between the six genotypes and confirmed the relationship between parents and hybrid which is promising genotype for sugarcane breeding program and production.

Start codon targeted polymorphism (SCoT) analysis was employed to distinguish 37 whipgrass (*Hemarthria compressa* L.) clones and assess the genetic diversity and population structure among these genotypes (Guo *et al.,* 2014). Shahlaei *et al.* (2014) demonstrated the efficiency of SCoT molecular marker for genetic diversity analysis of 10 tomato (*Lycopersicum esculentum* L.) accessesions, using 10 selected SCoT primers which generated 83 fragments; of which 30 (36.14%) were polymorphic. Twenty-four start codon targeted (SCoT) markers were used to assess genetic diversity and population structure of indigenous, introduced and domesticated ramie (*Boehmeria nivea* L. Gaudich.). A total of 155 genotypes from five populations were investigated for SCoT polymorphism, which produced 136 amplicons with 87.5% polymorphism (Satya *et al.,* 2015).

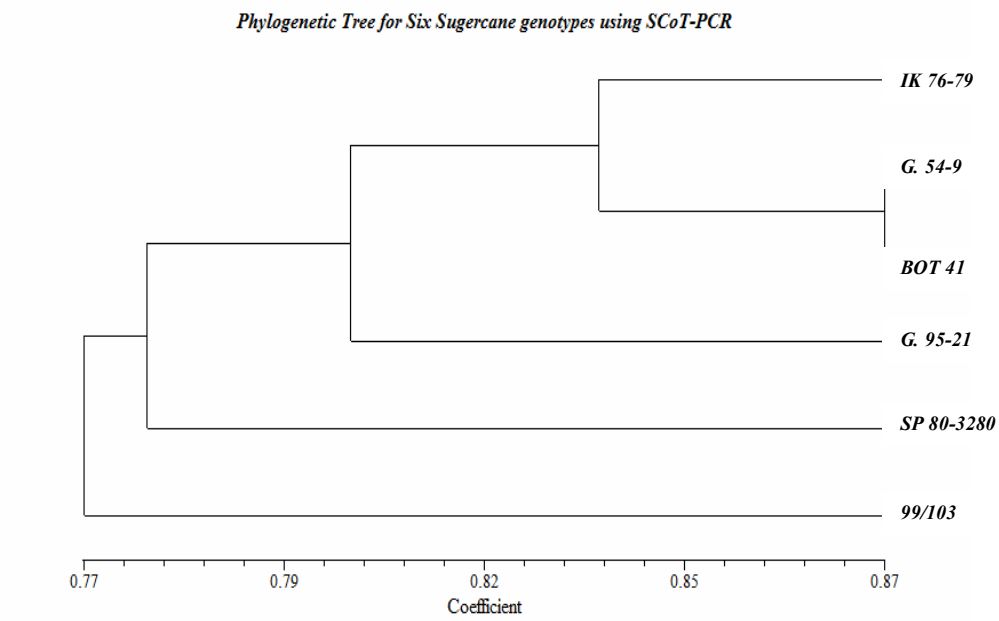

Fig. (2): Phylogenetic relationships for six genotypes of sugarcane using arithmetic averages; UPGMA.

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الملخص العربي

إستخدام الكشافات الجزيئية SCoT للتعرف على التباين الوراثي لستة أصناف من قصب السكر (*Saccharum* spp.)

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بالرغم من وجود العديد من التقنيات الوراثية المختلفة لدراسة الكشافات الجزيئية والتي تم إستخدامها لدراسة التراكيب الوراثية للنبات، فإن التقنية الحديثة والمسماة (SCoT) والتي تشير إلى تضاعف المناطق الخاصة والمحيطة بكودون البدء (ATG)، أثبتت فاعليتها في دراسة التباين والبصمة الوراثية. التقنية بسيطة، منخفضة التكلفة وتعطي نسبة عالية لتعدد الأشكال الخاصة بالمعلومات الوراثية الوفيرة، بالإضافة إلى إنها عامة في النبات. خلال هذه الدراسة، تم الحصول على ١٥١ حزمة متضاعفة بإستخدام عشرة بادئات (SCoT) مع ستة تراكيب وراثية من قصب السكر، ومن تلك الحزم هناك ٩٨ حزمة بنسبة (٦٤.٩%) تعدد أشكال. أيضاً هناك ٤٤ حزمة منفردة مقسمة ما بين الموجبة (تبعاً لوجود الحزمة) والسالبة (تبعاً لإختفاء الحزمة) والتي يعتقد إرتباطها ببعض الصفات الهامة في قصب السكر مثل نسبة السكر العالية والمقاومة للأمراض. أعلى نسبة تشابة ٨٧.٣٢% وجدت بين التركيب الوراثي رقم ٥ (G. 54-9) والتركيب الوراثي رقم ٦ (BOT 41) بإستخدام تحليل برنامج NTSYSpc 2.01b من خلال الـ UPMGA وذلك لإيجاد العلاقات الوراثية بين التراكيب المختلفة والممثلة في شكل شجرة النسب.