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Genetic Relationships among Some Stevia (*Stevia Rebaudiana* Bertoni) Accessions Based on ISSR Analysis

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Abstract: Stevia (*Stevia rebaudiana*) has many uses beside its traditional use as a sweetener. It has also been used as a medicine, cosmetic ingredient, pickling agent, and as a dentifrice. As a flavor enhancer, stevia helps to bring out the true flavors in cereals, breads, juices, candies, yogurt, and ice cream. Inter-simple sequence repeats (ISSR) molecular marker have been employed to determine the phylogenetic relationships among six stevia accessions using 7 primers. A total of 179 DNA fragments were amplified, of which 127 DNA fragment were polymorphic (71%). Thirty-three amplified fragments found to be positive specific markers, while 6 fragments were negative specific markers. ISSR primers S4, S5 and S11 showed 7, 2 and 5 specific markers, respectively and could be most specific for the accession St1. Genetic similarities among the six stevia accessions based on the ISSR data revealed that the accessions St4 and St6 were the most closely related (76.7% similarity). On the other hand, the two most distantly related accessions were St1 and St6 (30.1%). The results of the consensus tree indicated that the accession St1 was genetically distant from all studied accessions. The results suggests that the molecular genetic analysis using ISSR technique among different stevia accession provides accurate results as it revealed high degree of polymorphism. In conclusion, the present study shows that, the ISSR technique can be used in the breeding programs to determine and fingerprint the stevia accessions with high content of stevioside.

Key words: Stevia (*Stevia rebaudiana*), ISSR (inter-simple sequence repeats), molecular markers, phylogentic relationship

INTRODUCTION

Stevia rebaudiana Bertoni belonging to the family Compositae, is a non-caloric natural-source alternative to artificially produced sugar substitutes. It is one of 154 members of the genus *Stevia*, which produces sweet steviol glycosides[8,9]. Phillips[7] reported that stevia contains eight glucoside compounds. Stevioside is the most abundant glucoside produced. The extracts of these compounds may be up to 300 times sweeter than sugar[10]. Stevia can be used for many purposes; as medicinal plants and sweeteners. In Egypt, the gap between sugar production (1.757 million tons) and consumption (2.6 million tons) represents a serious problem, since it was estimated to be 0.843 millioin tons[1]. Nowadays, attention is concentrated upon using stevia in food industries, in order to close the gap between the production and consumption.

Molecular markers have been shown to be useful for diversity assessment in a number of plant species[12]. These markers, based on the polymerase chain reaction (PCR) technique, are the most commonly used for this purpose. Several molecular techniques are available for detecting genetic differences within and among cultivars[11]. An ISSR molecular marker technique permits the detection of polymorphism in microsatellites and inter-microsatellite loci without previous knowledge of DNA sequences[15]. ISSR could be amplified with oligonucleotide primers based on simple sequence repeats anchored at either the 3' or 5' end[4]. Wolfe[13] reported that inter-simple sequence repeats (ISSR) markers were originally devised for differentiating among closely related plant cultivars and have become extremely useful for studies of natural populations of plants, fungi, insects, and vertebrates.

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The objective of this study is to determine the genotype-spcific markers and phylogenetic relationships among some stevia accesstions based on ISSR markers.

MATERIAL AND METHODS

Plant Material: Six stevia accessions were kindly provided by Agriculture Research Station, Sabahia, Alexandria, Sugar Crops Research Institue (SCRI/ARC).

Methods:

Genomic DNA Extraction: A sample of about 0.1g of fresh young leaf tissues was used from each accession and ground into a fine powder using liquid nitrogen. Genomic DNA was extracted from the ground leaves using a modification of the BioFlux Kit.

ISSR-PCR Analysis: Seven ISSR primers out of fifteen based on dinucleotide, tetranucleotide or pentanucleotide repeats produced clear and reproducible fragments of all DNA stevia accessions (Table, 1).

The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L $MgCl_2$ (Promega), 100µM/L of each dNTP (Promega), 66ng/µl primer and 5 U/µl *Taq* polymerase in a 25µl volume. The amplification protocol was carried out according to Yao *et al*. (1999). The pre-reaction began with an initial denaturation at 94°C for 2 min, followed by 40 cycles of 10 s at 94°C, 30 s at 36°C, and 65 s at 72°C. The reactions were followed by a 10-min extension at 72°C and eventually stored at 4 ºC.

The amplified ISSR products were separated by electrophoresis on 1% agarose gel with 0.5x TBE buffer. After staining with ethidium bromide, banding patterns were visualized with a UV transilluminator.

Data Analysis: The banding patterns of seven ISSRs primers were scored as presence (1), or absence (0) bands by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. Similarity indices were calculated and consensus tree was developed based on the banding patterns of the 6 accessions ISSR analysis using SPSS statistical analysis program (Version 10). The genetic relationships among the 6 accessions, at the molecular level, were determined.

RESULTS AND DISCUSSION

Genotype-specific Markers Based on ISSR Primers: The results of amplified fragments (AF), specific markers (SM) for each accession of stevia using ISSR-PCR analysis are shown in Table (2) and Fig. (1). Seven primers out of fifteen ones produced a very high degree of polymorphism with a total of 179 reproducible fragments ranging from 17 (primer S4) to 31 fragments (primer S15). The results showed that three DNA amplified fragments were monomorphic in the six accessions and 127 amplified fragments were polymorphic. The highest percentage of polymorphism (93.1%) was recorded using the primer S12, while the lowest percentage (47.1%) was recorded using primer S4.

Accession-specific markers are shown in Tables (2 and 3). Thirty-three out of 179 (18.4%) fragments were found to be useful as genotype-specific markers. The highest number of specific markers was scored for the accession St1 (18 markers), while the lowest number of specific marker was scored for accessions St2 and St4 (1 marker). A number of 33 positive specific markers were scored for the presence of unique bands for the six accessions, while there were 6 negative specific markers for the absence of a common fragment. The highest number of accession-specific markers was generated by primer S4 (9 markers), while primers S5, S10 and S12 produced two specific markers for each. In conclusion, all ISSR primers used could produce polymorphic markers, to discriminate and identify the different accessions. Moreover, ISSR are considering a universal markers, which can be used in different species. This agreed with the results of McGregor *et al*.[5], since they concluded that ISSR and SSR techniques can individually identify each cultivar, but that techniques differ in the mean number of profiles generated per primer (or primer pair) per cultivar, referred to as Genotype Index (GI). These advantages of ISSR technique can be used in the breeding programs of stevia production with high quantities of stevioside. These advantages of ISSR technique can be used in the breeding programs of stevia production with high content of stevioside.

Genetic Similarity and Cluster Analysis Based on ISSR Analysis: Genetic similarities among the six stevia accessions were estimated according to the ISSR data. Table (4) showed that the most two closely related accessions were St4 and St6 with the highest similarity index (76.7%). On the other hand, the results indicated that the two most distantly related accessions were St1 and St6 with low similarity index (30.1%). The results of the consensus tree indicated that the dendrogram revealed one main group of six accessions

Table 1: Code and sequence of the eleven different ISSR primers.

No.	OligoName	Code	SEQUENCE
1	ISSR 844B	S3	5'-CTC TCT CTC TCT CTC TGC-3'
2	ISSR 17898A	S4	5'- CAC ACA CAC ACA AC -3'
3	ISSR 17898B	S5	5'- CAC ACA CAC ACA GT -3'
7	ISSR HB-10	S10	5'- GAG AGA GAG AGA CC -3'
8	ISSR HB-11	S11	5'- GTG TGT GTG TGT CC -3'
9	ISSR HB-12	S12	5'- CAC CAC CAC GC -3'
11	ISSR HB-15	S15	5'- GTG GTG GTG GC -3'

Table 2: Data obtained by ISSR analysis for the six stevia accessions, using seven ISSR primers.

Primer	TAF	PF	% PF	Landraces												TSM	Common bands
				St1		St2		St4		St5		St6		St7			
				AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM		
Pr.S3	22	17	77.3	7	1	6	1	3	1	4	1	1	0	1	1	5	0
Pr.S4	17	8	47.1	10	7	1	0	1	0	1	0	2	1	2	1	9	0
Pr.S5	30	15	50	7	2	6	0	5	0	4	0	3	0	5	0	2	2
Pr.S10	26	18	69.2	5	0	4	0	3	0	5	1	4	1	5	0	2	1
Pr.S11	24	17	70.8	7	5	3	0	3	0	5	1	3	0	3	1	7	0
Pr.S12	29	27	93.1	6	1	4	0	5	0	5	0	3	0	6	1	2	0
Pr.S15	31	25	80.7	9	2	6	0	1	0	7	2	1	1	7	1	6	0
Total	179	127	71	51	18	30	1	21	1	31	5	17	3	29	5	33	3

TAF= Total amplified Fragments, PF= Polymorphic fragments, TSM= Total specific markers, AF= Amplified fragments and SM= Specific marker

Table 3: Genotype-specific markers in six stevia accessions resulting from seven different ISSR primers.

Accession	Marker type		Total
	Positive	Negative	
St1	S3-522 S4-760, 672, 593, 464, 322, 292, 233 S5-467, 208 S11-584, 414, 206 149, 42 S12-144 S15-1100,557	S11-456, 227	20
St2	S3-1196	0	1
St4	S3-1297	0	1
St5	S3-273 S10-285 S11-705 S15-1044, 601	0	5
St6	S4-403 S10-172 S15-660	S5-534 S10-368 S15-675	6
S7	S3-248 S4-440 S11-173 S12-756 S15-715	S11-327	6
Total	33	6	39

Table 4: Similarity indices for the six stevia accessions based on their banding patterns with ISSR.

Accession	St1	St2	St4	St5	St6
St2	.603				
St4	.397	.658			
St5	.384	.616	.712		
St6	.301	.589	.767	.671	
St7	.342	.630	.726	.685	.685

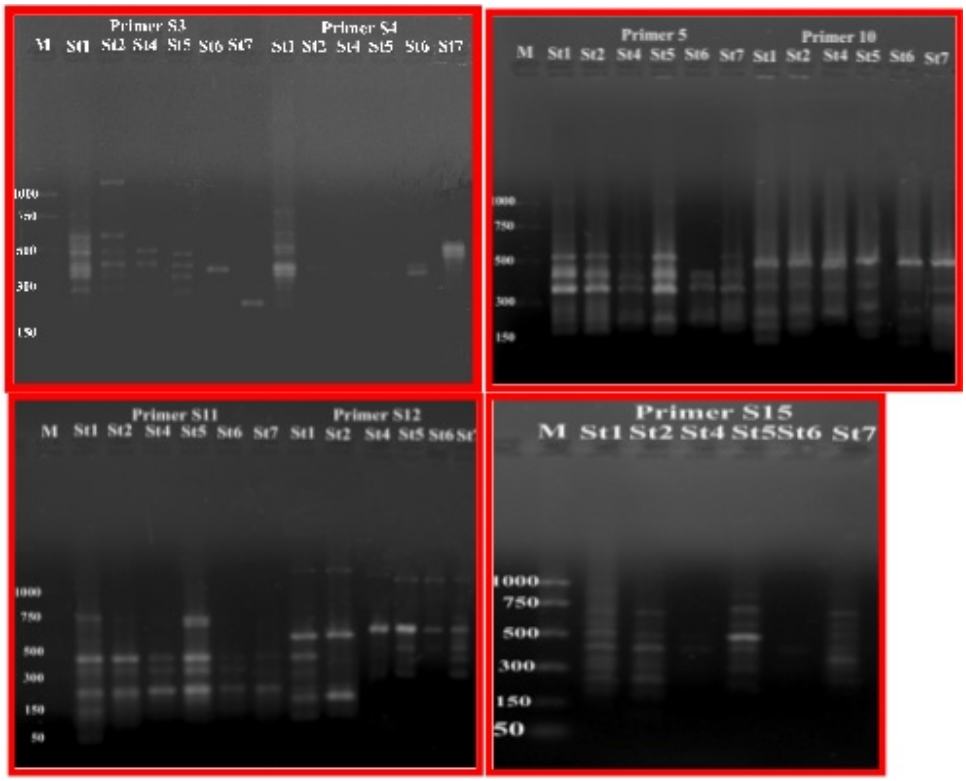

Fig. 1: ISSR-PCR banding patterns of DNA for six stevia accessions (St1, St2, St4, St5, St6 and St7) using Seven ISSR primers. M refers to 250 bp Ladder.

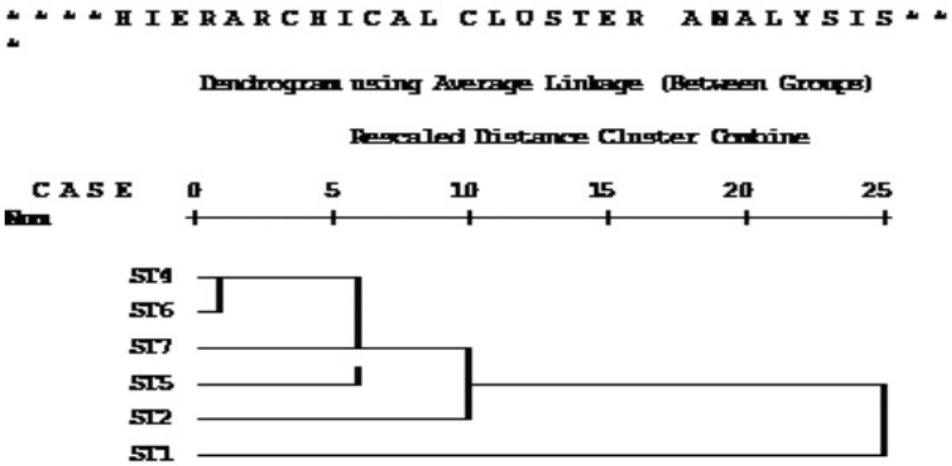

Fig. 2: Consensus tree for six stevia accessions developed on the basis of their banding patterns with ISSR primers.

including two subgroups. Subgroup 1 included 4 accessions (St4, St6, St7 and St5) and subgroup 2 included accession St2 only. The remaining accession (St1) was genetically distant from all accessions (Fig., 2).

The results confirmed that ISSRs profiling is powerful method for identification and molecular classification, which agreed with Hassan[3], Pharmawati *et al*.[6] and Gobert *et al*.[2]. In conclusion, the molecular genetic analysis using ISSR technique among different stevia accessions provided accurate results as it revealed high degree of polymorphism.

REFERENCES

1. Alaam, A.I., 2007. Sugar crops council: Future view. The Proceeding of Thirty-eight Annual Conference, Egyptian Sugar Expertese Society. Hawamdia, Egypt.
2. Gobert, V., S. Moja, P. Taberlet and M. Wink, 2006. Heterogeneity of three molecular data partition phylogenies of Mints related to *M. piperita* (*Mentha; Lamiaceae*), Plant Boil. (Stuttg), 8: 470-485.
3. Hassan, A.H.M., 2005. Identification of molecular markers for some morphological and biochemical characters in some medicinal plants. M.Sc. Thesis, Ain Shams Univ., Fac. Agric.
4. Lin, H., H. Lü, X. Zou, X. Bi, D. Yan and C. He, 2006. Genetic characterizations of *Mactra veneriformis* (Bivalve) along the Chinese coast using ISSR-PCR markers. Aquaculture, 261: 865-871.
5. McGregor, C.E., C.A. Lambert, M.M. Greyling, J.H. Louw and L. Warnich, 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSSR, AFLP and SSR) in tetraploid potato (*solanum tuberosum* L.) germplasm. Euphytica, 113: 135-144.
6. Pharmawati, M., G. Yan and P.M. Finnegan, 2005. Molecular variation and fingerprinting of *Leucadendron* cultivars (Proteaceae) by ISSR markers. Ann. Bot. (Lond), 7: 1163-1170.
7. Phillips, K.C., 1998. Stevia: steps in developing a new sweetener: In T.H. grenby ed. Developments in Sweeteners. Elsevier Applied, London.
8. Robinson B.L., 1930. Contributions from the Grey Herbarium of Harvard University. The Grey Herbarium of Harvard University, Cambridge.
9. Soejarto D.D., A.D. Kinghorn and N.R. Fransworth, 1982. Potential sweetening agents of plant origin. J. Nat. Prod., 45: 590-599.
10. Tanaka, O., 1997. Improvement of taste of natural sweeteners. Pure Appl. Chem, 69: 675-683.
11. Vos, P., R. hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijtens, J. Pot, J. Peleman, M. Kuiper and M. Zabeau, 1995. A new technique for DNA fingerprinting. AFLP: Nucleic Acids Research, 23: 4407-4414.
12. Waugh, R. and W. Powell, 1992. Using RAPD markers for crop improvement. Trends Biotech., 10: 186-191.
13. Wolfe, A.D., 2005. ISSR techniques for evolutionary biology. Methods Enzymol., 395: 134-144.
14. Yao, Y., M. Ban, and J. Brandle, 1999. A genetic linkage map for *Stevia rebaudiana* . Genome 42: 657-661.
15. Zietkiewicz, E., A. Rafalski and D. Labuda, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-183.