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Assessment of Genetic Diversity and Hybrid Identification in Stevia Using Inter Simple Sequence Repeat (ISSR) Markers

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Abstract

Identification of intra-specific diversity is an essential condition for the analysis of genetic diversity. *Stevia rebaudiana* Bertoni belonging to the family Compositae, has a long and successful history of use as a commercial sweetening agent. Stevia is a self-incompatible plant. Originated from a temperate country Paraguay, stevia is considered as an introduced species thus the number of stevia accessions available in Malaysia is still small and limited. Hybridization through *Trigona*-assisted pollination has successfully produced seven putative stevia F_1 hybrids. In this study, 17 stevia accessions and introductions, mostly from Paraguay and different parts of Malaysia including seven putative stevia hybrids were assayed by inter-simple sequence repeat (ISSR) markers to differentiate and explore their genetic relationships. A total of 332 clear bands were generated, out of which 275 (82.8 %) were polymorphic. The total number of markers varied from 6 to 18 with a mean of 10 markers per primer. The number of polymorphic markers for each primer varied from 3 (IS34/2) to 17 (IS55) with a mean of 8 polymorphic markers per primer. The amplified product size ranged from 50125 to 5000 bp. The PIC values ranged from 0.16 (IS34/2) to 0.5 (IS19, IS55, IS50, IS70 and IS78), with a mean PIC value of 0.43. The Jaccard's similarity coefficient values ranged from 0.375 to 812 with an average of 0.54. A dendrogram constructed based on the UPGMA clustering method, revealed two major Groups (A and B). Group-A showed segregated Bertam while Group B was further segregated into seven subgroups consisted of the remaining accessions studied. The present findings unarguably suggest extending the scope of detecting and quantifying the prevalent genetic diversity existing at the molecular level in the stevia accessions and the putative hybrids. To our knowledge this is the first report on the characterization stevia accessions and developed putative stevia hybrids in Malaysia based on thirty two primers. ISSRs have the potential to distinguish closely related accessions based on the patterns of their amplicons. High polymorphism obtained indicates ISSR is an efficient technique or evaluating genetic diversity in stevia. The present study also elucidates the utility of ISSR markers as an efficient tool to screen putative hybrids obtained from *Trigona*-assisted pollination.

Key words: Stevia, genetic diversity, hybrid, ISSR, *Trigona*-assisted pollination

INTRODUCTION

Assessment of genetic variability, diversity and intra-relationships in a species is essential for varietal improvement programme. Traditionally, morphological markers for genetic diversity have been employed vastly by breeders. But the number of available morphological descriptors are very limited and in vogue for characterisation purposes. Thus, a large number of molecular markers have been developed in the recent years in aim of overcoming the limitations associated with morphological markers.

Stevia rebaudiana Bertoni is becoming a popular crop due to the sweet properties contained in the leaves (Brandle, 2004). It is native to Paraguay but now has been widely cultivated in various countries including Japan, Taiwan, Korea, Thailand and Indonesia for natural sweetener purposes. The highlight on stevia is as the main source of non-caloric sugar, a great alternative to synthetically produced sugar substitute with an advantage of being 300 times sweeter than sugarcane sugar (Singh & Rao, 2005). It is self-incompatible and insect pollinated. The introduction of stevia into Malaysia was as early as 1970s. Although stevia is seen as having a potential to become a viable crop, the number of suitable stevia varieties in Malaysia is still lacking. Currently, practical and suitable variety is still unavailable to adapt well under local environmental condition. The main breeding strategy for stevia to broaden the genetic diversity is through hybridisation. However, hybridisation through manual crossing is very laborious and difficult. Therefore, this study has been initiated to develop F_1 stevia hybrids through Trigona-assisted pollination. The technology to develop Trigona-assisted hybridisation has now been successfully proven. A line of F_1 stevia hybrids and its genetic variants have been produced.

Hybrids are usually identified based on their

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morphological characteristics. However, morphological characteristics are obviously insufficient for the identification of F_1 hybrids; hence analysis at the DNA level using molecular method is required. Alternatively, molecular markers method allows rapid identification of plant genotypes (hybrids, clones, somaclonal variants and cultivar) with high resolving power (Perry, 2004) and low labor cost (Reddy et al., 2002). Moreover, these markers are not stage and tissue specific and most importantly are not affected by the environment.

Molecular markers such as RFLP, SSR, ISSR and AFLP, used to detect DNA polymorphism are used vastly for identification of artificial and natural hybrids in many plant species (Wolfe et al., 1998; Rajora and Rahman, 2003; Ruas et al., 2003; Shasany et al., 2005). Amongst the various DNA-based markers available, inter simple sequence repeat (ISSR) has been shown to give rapid, simple, reproducible and inexpensive means in molecular taxonomy, conservation, breeding and genetic diversity analysis. ISSR markers amplify regions between adjacent and inversely oriented microsatellites using di-, tri-, tetra- and penta-nucleotide SSR primers with the advantage that the information of the target DNA sequence is not required. The amplification depends on the variation, motif, frequency of SSRs that changed due to natural crossing and/or mutation induction (Carvalho et al., 2008). Thus, ISSR is excellent for fingerprinting and characterisation of species and inter-specific hybrids (Carvalho et al., 2008; Kumar et al., 2009).

In the present study, we investigated the utility of ISSR markers in genetic diversity study of stevia accessions and putative hybrids.

MATERIALS AND METHOD

Plant Materials

A total of 24 stevia accessions (inclusive of 7 stevia putative hybrids) were used in the study (Table 1).

DNA Extraction

Total genomic DNA was extracted rom young fresh stevia leaves using CTAB method (Doyle and Doyle, 1990). Extracted DNA samples were run on 0.9% agarose gel for confirmation of extracted DNA followed by quantification and confirmation of its good quality. DNA samples were then kept at -20°C until use.

ISSR Analysis

ISSR analysis was performed with 32 primers procured from 1st Base Laboratories (Table 3). PCR reaction was performed in final volume of 50 µl containing 1X Mytaq PCR Master Mix (Bioline, UK), 2 µM/reaction of ISSR primer and 250ng of template DNA. The PCR was performed in Eppendorf Mastercycler® thermocyler. The PCR programme comprised of 40 cycles and the PCR tubes were subjected to the thermal profile given in Table 2. Following amplification, the PCR products were loaded on 1.2% agarose gel (HydraGene™ molecular grade), which was prepared in 1X TAE buffer containing 1.5 µg/ml o the Ethidium Bromide. The amplified products were electrophoresed for 1 hour at 100 V. After separation, the gel was viewed under UV trans-illuminator.

RAPD Profile Analysis

The screened primers that gave bands were used to amplify the DNA of all the 24 stevia accessions. The ISSR profile was characterised based on its banding pattern. ISSR bands as viewed from the gels after electrophoresis and staining were designated on the basis of their molecular sizes (length of polynucleotide amplified). The screened primers that gave bands were used to amplify the DNA of all the 24 stevia accessions. The ISSR profile was characterised based on its banding pattern. ISSR bands as viewed from the gels after electrophoresis and staining were designated on the basis of their molecular sizes (length of polynucleotide amplified).

These ISSR markers were converted into a matrix of binary data, where the presence of the band corresponded to the value of 1 and the absence to value 0. The scores (0 or 1) for each band were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient (Jaccard, 1908). Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmettic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendogram, depicting the relationships of the accessions using computer program NTSYS pc version 2.1 (Rohlf, 2000).

Diversity for each marker was determined using the polymorphic information content (PIC) calculated according to (Anderson et al. (1993) as,

PIC = 1- (F x F) + (E x E)
Where, F = No. of bands present
E = No. of bands absent

Thirty two ISSR primers obtained from 1st Base Laboratories were employed or genetic diversity analyses.

RESULTS AND DISCUSSIONS

Molecular Characterisation

Out of the 42 ISSR primers screened, positive banding patterns were obtained with 32 primers (Table 3). A total of 332 loci were amplified out of which 275(82.8%) were polymorphic and 57 were monomorphic (17.2%) (Table 4). The number of polymorphic markers for each primer ranged from 3 to 17 with a mean of 8 markers per primer. The highest polymorphic loci was obtained with primer IS55 (17) and lowest was IS34/2 (3). The amplified product size ranged between 150 to 5000 bp. PIC value ranged from 0.16 (IS34/2) to 0.5 (S19, IS55, IS50, IS70 and IS78), with mean PIC value of 0.43. The discrimination power of each locus was estimated by the

Table 1. Description of stevia accessions/ varieties and hybrids used in the study and their collection sites

No	Accession/Variety	Origin	Area	Latitude	Longitude	Altitude (m)	Description
1	Bangi	Malaysia	Bangi	2.91984	101.780868	69	Existing in germplasm collection
2	Nilai	Malaysia	Nilai	2.802455	101.798871	33	Existing in germplasm collection
3	Rawang	Malaysia	Rawang	3.320482	101.575924	37	Existing in germplasm collection
4	Langat	Malaysia	Langat	3.113117	101.815673	52	Existing in germplasm collection
5	Mergong	Malaysia	Mergong	6.131769	100.344176	5	Existing in germplasm collection
6	Bertam	Malaysia	Bertam	2.281558	102.195796	26	Existing in germplasm collection
7	Taman Pertanian	Malaysia	Taman Pertanian	3.096541	101.51189	28	Existing in germplasm collection
8	Souq Bukhori	Malaysia	Bukhari Market	31.519297	74.31519	213	Existing in germplasm collection
9	MS007	Malaysia	MARDI, Serdang	3.004202	101.700176	50	Existing in germplasm collection
10	MS012	Malaysia	MARDI, Serdang	3.004202	101.700176	50	Existing in germplasm collection
11	Exotic	Malaysia	Kuantan	3.79922	103.277454	6	Seedlings from Exotic Nursery, Kuantan
12	MNQ	Malaysia	Kuantan	3.797305	103.266676	6	Seedlings from MNQ Nursery, Kuantan
13	Rasa Sayang	Malaysia	Kuantan	3.808576	103.330432	10	Seedlings from Rasa Sayang Nursery Kuantan
14	Kelantan	Malaysia	Wakaf Bharu	6.118858	102.198715	10	Seedlings from Kelantan
15	Native	Paraguay	Lambare	-25.345815	-57.60991	115	Seeds from Paraguay. Also known as Criole
16	Morita III	Paraguay	Lambare	-25.345815	-57.60991	115	Seeds from Paraguay
17	Eirete II	Paraguay	Lambare	-25.345815	-57.60991	115	Seeds from Paraguay
18	MS007 Hyb1	Malaysia	Kuantan	3.841391	103.302854	35	Derived from MS007
19	MS007 Hyb2	Malaysia	Kuantan	3.841391	103.302854	35	Derived from MS007
20	Eirete Hyb	Malaysia	Kuantan	3.841391	103.302854	35	Derived from Eirete II
21	Langat Hyb	Malaysia	Kuantan	3.841391	103.302854	35	Derived from Langat
22	Nilai Hyb1	Malaysia	Kuantan	3.841391	103.302854	35	Derived from Nilai
23	Nilai Hyb2	Malaysia	Kuantan	3.841391	103.302854	35	Derived from Nilai
24	Nilai Hyb3	Malaysia	Kuantan	3.841391	103.302854	35	Derived from Nilai

Table 2. Thermocycling profile

Cycle step	Temperature (°C)	Time
Initial denaturation	94	5 mins
Denaturation	94	1 min
Annealing	X	1 min
Extension	72	1 min
*Repeat for 39 cycles		
Final extension	72	10 mins
Hold	4	∞

X: Based on primer pair optimum annealing temperature

Table 3. List of ISSR primers used including their nucleotide sequence and respective annealing temperature* used to screen DNA polymorphism from stevia accessions and hybrids samples

Primer	5'-3'Primer Sequence	Tm (°C)	Total fragments	Approx. fragment size (kb)
Di-nucleotide				
IS12	(AG)8T	45	8	500-1600
IS19	(CT)8T	51	9	200-2500
IS20	(CA)8A	51	8	200-1400
IS21	(CA)8G	51	6	450-1600
IS23	(GT)8C	55	10	400-2000
IS25	(TC)8A	55	10	300-2500
IS30	(AC)8C	55	9	300-1400
IS34/1	(GA)8CT	45	13	200-2000
IS34/2	(GA)8TT	45	8	500-1500
IS42/2	(AC)8TG	48.6	12	300-2200
IS44/1	(AC)8CT	52.9	6	700-1800
IS44/2	(AC)8TT	52.9	7	300-1300
IS54	(AG)8C	55	12	400-2500
IS55	(AG)8A	55	18	200-1600
IS56	(TC)8C	51	9	300-1600
IS57	(GA)8CT	51	12	400-2100
IS83	(AG)5TT	46.9	8	350-1400
IS85	(CT)5CACC	51	12	350-2500
IS90	(AG)8G	55	9	300-2300
S4	(CA)6AC	36	11	300-1400
S5	(CA)6GT	36	11	350-1500
S10	(GA)6CC	36	9	300-1300
S11	(GT)6CC	36	11	300-2000
UBC836	(AG)8CA	46	13	400-2500
Tri-nucleotide				
IS50	(GAA)6	54.2	17	200-5000
IS52/1	(TCC)5AC	48.6	12	200-5000
IS52/2	(TCC)5GT	48.6	11	400-2000
IS70	(GAA)6	52.9	9	150-3000
IS78	(AGA)7	48.2	15	250-3000
IS94	(ATG)6	55	8	300-900
S15	(GTG)3GC	36	8	400-1500
Tetra-nucleotide				
S12	(CACA)3GC	36	11	300-1450
	Total = 332			

Primers synthesized from 1st Base Laboratories Sdn Bhd. Malaysia

PIC (polymorphism information content) value.

Genetic similarity estimates based on ISSR banding patterns were calculated using method Jaccard's coefficient analysis (Table 5). The Jaccard's pairwise similarity coefficient values ranged from 0.375 (Souq Bukhori and MNQ) to 0.812 (Rawang and Nilai) with an average of 0.54, for a single primer based ISSR patterns. The cluster constructed through NTSYS (2.1 pc) presented in the form of dendogram are shown in Figure 2. The cluster analysis revealed two groups (A and B). Group B was further divided into two seven subgroups (1-7). Subgroup 1 consisted of (MS007, MS012, Bangi, Rawang, Nilai, Langat, T. Pertanian). Subgroup 2 consisted of (Native, Kelantan, Exotic). Subgroup 3 contained (Mergong, Eirete II, Morita III). Four out of the seven putative hybrids were grouped in subgroup 4 (Langat Hyb1, Nilai Hyb3 Nilai Hyb1, Nilai Hyb2). Another three putative hybrids (Eirete II Hyb, MS007 Hyb1, MS007 Hyb2) and Rasa Sayang were contained in subgroup 5. MNQ was in subgroup 6 and subgroup 7 was Souq Bukhori. Group B only consisted of a segregated Bertam. Group A and B showed 44% between group similarities. The seven subgroups showed 45% within group similarity.

The thirty two ISSR primers that were selected for analysis generated 275 ISSRs, with an average 10 products/primer and size ranged from 150 to 5000 bp. Cluster analysis indicated the segregation of different accessions studied at 0.44 similarity coefficients. The acquired data in this investigation proved the significance o ISSR in the discrimination among the studied accessions, which is in concordance with the results of Heikal et al., (2008); Neha et al., (2016); Saini et al., (2004); Blair et al., (1999); Lakshmi et al., (2002); Yadav et al., (2007). ISSR products are usually dominant markers and are inherited in simple Mendelian fashion, therefore verified as to be taxonomically and evolutionary useful at all taxonomic levels especially at the intra- and inter- specific levels (Demeke and Adams, 1994; Nkongolo et al., 2002).

Different levels of genetic polymorphism among 24 stevia accessions and putative hybrids were revealed. It was

Table 4. egree of polymorphism and polymorphic information content for ISSR primers

Primer	TNF	NPF	NMF	NUF	PP	PIC
Di-nucleotide						
IS12	8	7	1		87.5	0.47
IS19	9	7	2		77.78	0.5
IS20	8	6	2		75	0.47
IS21	6	5	1		83.33	0.49
IS23	10	9	1	1 (6)	90	0.49
IS25	10	10	0		100	0.49
IS30	9	7	2	1(10)	77.78	0.35
IS34/1	13	13	0		100	0.33
IS34/2	8	3	5		37.5	0.16
IS42/2	12	10	2		83.33	0.49
IS44/1	6	4	2	1(12)	66.67	0.47
IS44/2	7	6	1		85.71	0.44
IS54	12	10	2		83.33	0.46
IS55	18	17	1	2(13,18)	94.44	0.5
IS56	9	7	2		77.78	0.4
IS57	12	12	0		100	0.48
IS83	8	5	3		62.5	0.38
IS85	12	9	3		75	0.48
IS90	9	8	1		88.89	0.44
S4	11	8	3		72.73	0.33
S5	11	7	4		63.64	0.48
S10	9	6	3		66.67	0.31
S11	11	9	2		81.82	0.36
UBC836	13	12	1		92.31	0.46
Tri-nucleotide						
IS50	17	16	1	2 (13,14)	94.12	0.5
IS52/1	12	12	0		100	0.48
IS52/2	11	10	1	1(8)	90.91	0.47
IS70	9	9	0		100	0.5
IS78	15	15	0	1(8)	100	0.5
IS94	8	5	3		62.5	0.41
S15	8	6	2		75	0.42
Tetra-nucleotide						
S12	11	5	6		45.45	0.35
Total	332	275	57	9	2591.69	13.86
Average/Primer	10^{MR}	8.59^{EMR}			80.99	0.43

NTF: Number of total fragments scored, NPF: number of polymorphic fragments, NMF: number of monomorphic fragments, NUF: number of unique fragments, PP: percentage of polymorphism, PIC: polymorphic information content, MI: marker index
Values in parantheses of unique bands denote number of accession given in Table 1.

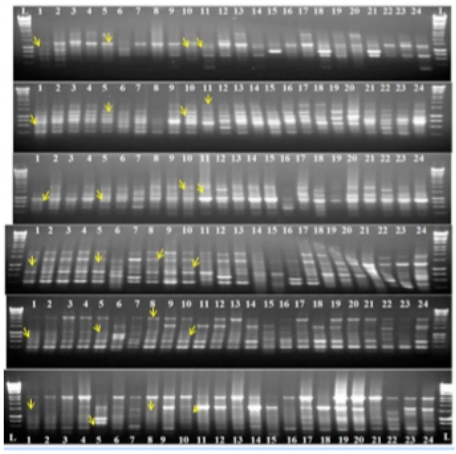


Figure 1. ISSR amplification profile of stevia genotypes with ISSR primer IS25, IS34/1, IS52/1, IS57, IS70 and IS78 respectively. L- 1kb DNA ladder (Bioline, UK) Lane 1-24 numbers refer to accessions designated in Table 1. *Arrows indicate mother-plant genotype specific markers*

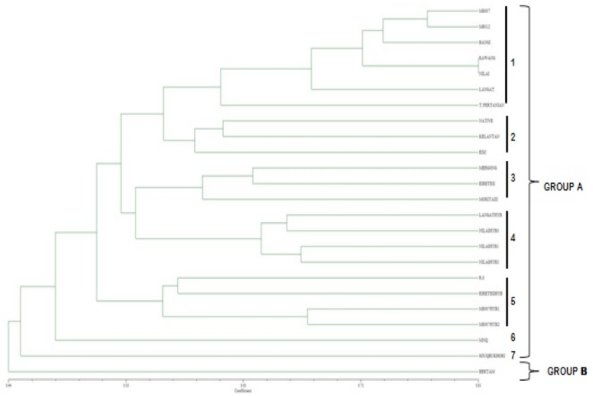
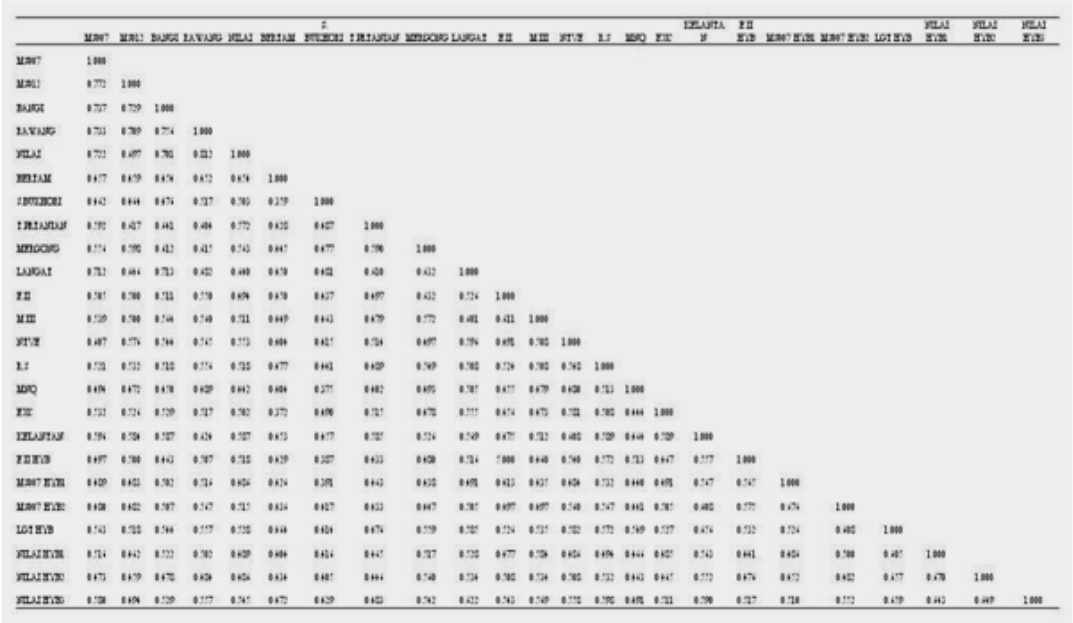


Figure 2. Dendogram illustrating genetic relationship among 24 stevia accessions, produced by UPGMA cluster tree analysis (NTSYS pc) calculated from 332 ISSR markers generated from 32 ISSR primers

noticed that different primers gave different levels of polymorphism (Table 4). This can be explained on the presumption that homology of sequences o primers with the complementary sequences present in stevia accessions genome. ISSR technique is a common and well-proven tool in genetic studies and a suitable system for detecting total genetic variation and it's partitioning within and among populations. This technique has been effectively used in a variety of taxonomic and genetic diversity studies (He et al., 2007; Li et al., 2008; Lee and Jin, 2008; Huang et al., 2009; Vijayanand et al. 2009; Dos Santos et al., 2011. Stevia showed a high percentage of genetic differentiation. The level of genetic diversity detected by ISSR are in overall agreement with studies by Han et al., (2007); Manikemalai et al. (2007); Boussaid et al., (2007). ISSR being a multi-locus marker with the simplest and fastest technique has been successfully employed for the determination of intra-species genetic diversity in several plant species (Reddy et al., 2002;Zietkiewiez et al. 1994; Blair et al., 1999; Nagaraju et al., 2002).

The calculated PIC values were based on the probability that the two unrelated genotypes amplified from the test population will be placed into different typing groups. Apparently it is an index to determine how many alleles a certain marker has and in what way those alleles divide. High PIC value indicates enormous heterozygosity which is associated with the degree of polymorphism (Zimmer and Roalson, 2005). Presently a good range of PIC value was observed which indicated significant genetic diversity among stevia accessions.

Table 5. Dendogram illustrating genetic relationships among 24 stevia genotypes produced by UPGMA cluster tree analysis (NTSYS pc) calculated from 332 ISSR markers generated from 32 ISSR primers



CONCLUSION

The present study showed that ISSR technique is quick and reliable which put ISSR as a preferred method for breeders in assessing the genetic diversity for utilisation in breeding programmes. ISSR is excellent at detecting high level of genetic diversity and relationship in stevia accessions and therefore can be further established as an essential basis to aid in stevia future conservation and breeding programmes. As a conclusion, the information gathered from this study is a valuable tool towards proper breeding strategies in producing stevia with high sweetener content.

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