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Identification and functional annotation of expressed sequence tags based SSR markers of *Stevia rebaudiana*

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Abstract: *Stevia rebaudiana* Bertoni, a perennial herb belonging to the family Asteraceae, is a tropical as well as subtropical plant of medicinal importance. Despite being an important medicinal plant and in fact proving a boon to diabetics, it has remained almost ignored by molecular biologists. A systematic molecular evaluation of stevia germplasm and molecular breeding work are sparsely reported mainly due to the limited specific molecular markers available. Expressed sequence tags (ESTs) are a valuable resource for developing simple sequence repeats (SSRs). In this study, 5548 stevia ESTs sequences from leaf tissues were retrieved from the NCBI database. Clustering and assembly of these ESTs resulted in a nonredundant set of sequences, i.e. a total of 471 contigs and 3845 singleton ESTs. Out of these 471 contigs and 3845 singletons, 168 SSRs were identified. Dinucleotide SSR is the dominant repeat type (62.5%), followed by trinucleotide (37.5%). Annotation analysis revealed that putative function could be assigned to 82.2% of EST-SSRs. After using Primer3 software, 18 primers were custom synthesized from the SSR containing 18 singletons and generated 61.11% polymorphism. In silico mined markers with functional annotations, in the present study, facilitated genome analysis in stevia, which had not been performed previously. Additionally, the EST-SSRs will be used for molecular work in related plant species since they generally exhibit cross species transferability, making further work cost effective and simple.

Key words: EST, functional annotation, genetic diversity, SSR, *Stevia rebaudiana*

1. Introduction

Expressed sequence tags (ESTs) are created by partially sequencing randomly isolated gene transcripts that have been converted into cDNA (Adams et al., 1992). Any actively growing tissue can be used to generate cDNAs from mRNA and sequenced to generate ESTs that are assembled into a nonredundant set of sequences (contigs and singletons). Recent research has revealed that ESTs are a potentially rich source of SSRs that reveal polymorphism.

SSRs repeat motifs of 1–6 bp in length are hypervariable and widely spread in eukaryotic genomes (Vosman and Arens, 1997; Rallo et al., 2000). SSRs are reproducible, multiallelic, co-dominant, abundant, and cover the whole genome and have become markers of choice for diversity analysis and genome analysis (Morand et al., 2002; Zeid et al., 2003; Yi et al., 2006; Jiao et al., 2012; Wijarat et al., 2012).

However, the traditional genomic library dependent approach for SSR marker development is time consuming and cannot be carried out in a laboratory without much of an infrastructure. Over the last 10–15 years there has

been continuous progress on EST sequencing work in different laboratories that has led to in silico data mining for ESTs and their applications in various molecular biology projects. With the advances in bioinformatics, it is possible to mine and analyze large-scale EST datasets efficiently and exhaustively in various organisms (Ewing et al., 1999; Ronning et al., 2003). An in silico database offers the opportunity to identify SSRs in ESTs through data mining and provides a simple and cost efficient approach for the development of SSR markers in plants (Gupta and Varshney, 2000). Currently there are near 20 million ESTs in the NCBI public collection-dbEST database (<http://www.ncbi.nlm.nih.gov/dbEST/>).

EST-SSRs have been reported in several plant species, such as cauliflower (Vaidya et al., 2012), walnut (Zhu et al., 2009), watermelon (Verma and Arya, 2008), citrus (Chen et al., 2006), sugarcane (Pinto et al., 2004), grapes (Scott et al., 2000), cereal species (Varshney et al., 2002), and bread wheat (Gupta et al., 2003). EST-derived markers have also been well documented in marker assisted selection that involves mainly the identification of QTL of important

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traits, genetic mapping, genetic diversity, and comparative genomics (Liu et al., 2005; Alwala et al., 2006; Miklas et al., 2006; Cloutier et al., 2009; Durand et al., 2010; Kumar et al., 2011; Vaidya et al., 2012).

Stevia rebaudiana is an important medicinal plant with antibacterial, antifungal, antiinflammatory, antiviral, antiyeast, cardiotoxic, and diuretic properties. The property of this species that has attracted attention is the intense sweet taste of its leaves and aqueous extracts. Basically, stevia is distinguished by the presence of sweet diterpene glycosides: rebaudioside-A, rebaudioside-C, stevioside, and dulcoside in its leaf tissue (Ahmed and Dobberstein, 1982). A total of nine sweet diterpenoid glycosides have been isolated from *S. rebaudiana*, namely: stevioside, steviolbioside, dulcoside-A, and rebaudioside-A, -B, -C, -D, -E, and -F (Kennelly, 2002; Starratt et al., 2002; Dacome et al., 2005). Stevioside is the major component but it has an unpleasant bitter aftertaste (Ishima and Katayama, 1976; Kinghorn and Soejarto, 1985). However, rebaudioside-A, normally present in lower amounts (25% to 45% of stevioside content) in leaves, does not have bitter aftertaste and has a sweetening power 1.2 to 1.6 times higher than that of stevioside. Therefore, the breeding objectives can be set for stevioside and rebaudioside-A content for this medicinally important plant. Earlier, Heikal et al. (2008) employed ISSR markers to determine the phylogenetic relationships among six stevia collections and Yao et al. (1999) first constructed its genetic linkage map. However, the genetic studies on stevia are chronically lagging behind compared with other economically important plant species.

To date, publically available molecular markers such as SSRs in stevia are limited, which has significantly hampered genome analysis and molecular breeding work. Therefore, the present study was undertaken with the following objectives: 1) Identification of SSRs in the transcribed regions of the *Stevia* genome; 2) Function domain analysis. The present study is an initiative in the direction of development of functional stevia-specific microsatellite markers and the main aim of the study was for these EST-SSR markers to be efficiently used for genetic mapping studies of segregating stevia populations.

2. Materials and methods

2.1. Source plant material

A survey was conducted to collect diverse *Stevia rebaudiana* plants from all 12 districts of Himachal Pradesh (a state in Northern India). During the survey, it was found that stevia is present in a few pockets of only four districts of the state. The plants were collected from these four districts, viz., Kangra (Palampur, Institute of Himalayan Bioresource Technology (IHBT) and Dharamshala, from a local farmer) at an altitude of 733 m

above mean sea level (amsl), Hamirpur (Hamirpur, near Institute of Biotechnology and Environmental Science (Neri) and Herbal Garden of Beri) at an altitude of 738 m amsl, Bilaspur (from a local farmer) at an altitude of 673 m amsl, and Solan (Nauni, Dr Y S Parmar University of Horticulture and Forestry, and Chail) at an altitude of 1275 m amsl.

Additionally, stevia plants were collected from the Indian Agricultural Research Institute (IARI), New Delhi, at an altitude of 250 m amsl. All the collections used for the present study are cited in Table 1.

2.2. EST search by *Stevia* EST mining

EST sequences belonging to *Stevia rebaudiana* leaf cDNA were acquired from NCBI in their FASTA format from the EST database (www.ncbi.nlm.nih.gov/nucest). A total 5548 ESTs were retrieved and kept as a single text file constructed containing all the detected sequences.

2.3. Clustering and assembly of EST sequences

A total of 5548 redundant ESTs retrieved from NCBI were used to produce a nonredundant dataset by using the EGassembler webserver (Masoudi-Nejad et al., 2006). The repetitive elements including small RNA pseudo genes, LINEs, SINEs, LTR elements, vector sequences, organelle, and other interspersed repeats were masked by the software. The software automatically screens and cleans for various contaminants in the retrieved EST sequences. The server clustered and assembled the sequences into contigs and singletons using CAP3 (Huang and Madan, 1999) with the criterion of 80% overlap identity between one end of a default read to another end.

2.4. SSR identification for the assembled ESTs

The potential SSRs were detected in the assembled ESTs by submitting the sequences to a search tool, namely SSRIT (simple sequence repeat identification tool; www.gramene.org/db/searches/SSRtool) (Temnykh et al., 2001). The sequence search for SSRs conducted by SSRIT was carried out by setting the search parameters to identify at least five repeat units SSR of maximum ten base pairs. The software needs to put input as a FASTA format sequence in which SSRs are to be found and the output file comprises the sequence name in which the SSR is detected, repeat motif of the SSR, number of times it is repeated, start and end of the repeat, and length of the sequence. The SSR motifs obtained as output of SSRIT were thoroughly counted for the number of repeats and their frequency and distribution were also recorded.

2.5. EST-SSR similarity search or BLASTx analysis

The frequency and nature of amino acids generated from the trinucleotide repeat motifs were studied because each trinucleotide motif codes an amino acid that has putative roles in the biological activity of protein molecules. To find out the putative functions of the EST SSRs, the

Table 1. List of 16 *Stevia rebaudiana* collections used in the present study for EST-SSR analysis.

S. no.	Denoted as	Place of collection	District
1.	Parent A	Nauni, Y S Parmar UHF	Solan
2.	Parent B	Nauni, Y S Parmar UHF	Solan
3.	St-1	Palampur, IHBT	Kangra
4.	St-2	Dharamshala, from a local farmer	Kangra
5.	St-3	Neri, Research station	Hamirpur
6.	St-4	Beri, Herbal Garden	Hamirpur
7.	St-5	Bilaspur, from a local farmer	Bilaspur
8.	St-6	New Delhi, IARI	New Delhi
9.	Nst-1	Nauni, Y S Parmar UHF	Solan
10.	Nst-2	Nauni, Y S Parmar UHF	Solan
11.	Nst-3	Nauni, Y S Parmar UHF	Solan
12.	Cst-1	Chail	Solan
13.	Rst-1	Replication cutting of St-1	Kangra
14.	Rst-2	Replication cutting of St-2	Kangra
15.	Rst-5	Replication cutting of St-5	Bilaspur
16.	Rst-6	Replication cutting of St-6	New Delhi

corresponding EST sequences were compared with the UniProt database (<http://www.uniprot.org/>) using the BLASTx program, assuming an E value of $\leq 1E - 5$ as a significant criterion of homology.

2.6. Functional domain analysis

Using PROSITE from Expasy tools (<http://prosite.expasy.org/prosite.html>) functional protein domains of the sequences containing SSRs were searched.

2.7. Primer design and polymerase chain reaction (PCR) analysis

Using the Primer3 software (www.frodo.wi.mit.edu/primer3) 18 primers were custom synthesized from the SSR containing 18 singletons randomly chosen from a total of 3845 singletons and these primers were used for the amplification of isolated DNA of collections of *Stevia rebaudiana*.

The PCR protocol was standardized to amplify the genomic DNA of stevia plants using EST-SSR primers. PCR was carried out in the presence of 60 ng of genomic DNA, 1X PCR buffer, 2.2 mM MgCl₂, 1 mM dNTPs, 3.0 μM of each primer, and 1 Unit 94 kD Taq DNA polymerase (all chemicals from Genei, Bangalore, India). The PCR amplification was performed in an automated thermal cycler (Applied Biosystems, USA) programmed for 5 min initial denaturation at 95 °C, 40 cycles of 1 min denaturation at 94 °C, 1 min annealing according to T_m for each primer and 2 min extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. The PCR

amplified products were subjected to electrophoresis in a 2% agarose gel at a constant voltage of 80 V. After electrophoresis, the gels were stained with 0.5 μg/mL ethidium bromide and a photograph taken under UV light in Gel Documentation system (Syngene, USA).

2.8. Data analysis

Two different programs, NTSYSpc ver. 2.02h (Rohlf, 2000) and DARwin5 ver. 5.0.155 (<http://darwin.cirad.fr/darwin>) (Perrier et al., 2003), were used to study the relatedness of the diverse *Stevia* plants. The data on band position on the gel were recorded by assigning '0' for the absence of band and '1' for the presence of band. After that, a dendrogram, similarity matrix tables, and rooted trees were constructed by using the UPGMA function of both programs.

Genetic diversity, defined as the polymorphism information content (PIC; Anderson et al., 1993), was used to measure allelic diversity at each locus. PIC values were calculated as follows:

$$PIC = 1 - \sum_{i=1}^k p_i^2,$$

where k was the total number of alleles detected for a microsatellite (SSR) marker and p_i was the frequency of the i th allele in the set of genotypes analyzed.

3. Results

3.1. NCBI database search

A total of 5548 EST sequences belonging to stevia were obtained from NCBI and used for further computations and analysis.

Out of the 5548 ESTs, 471 contigs were assembled and 3845 singletons were recorded that showed no overlap with any ESTs. After assembly, the whole dataset was reduced to 4316 sequences, presented in Table 2, which showed 22.2% data redundancy by using EGassembler. These nonredundant sequences were reported to have 168 EST-SSRs in which 28 EST-SSRs were reported by contigs and remaining 140 were from singletons. After assembly, a nonredundant group of ESTs became nonredundant, which included contigs and singletons, and these nonredundant ESTs were used for the SSR search tool.

3.2. SSRIT search

The SSRIT search for microsatellites or SSRs detected a total of 168 SSRs. Analysis of the detected SSRs revealed that all of them represented di- and trinucleotide repeats. From Table 3 it is seen that dinucleotide SSR is the dominant repeat type (62.5%), followed by trinucleotide (37.5%). Among the trinucleotide repeat motifs the dominant repeat motif is ACC (12.7%), followed by ATC (11.1%). Table 4 clearly depicts that among dinucleotide repeats the most frequent repeat was CT/TC (32.3% followed by AT/TA (27.6%), while the least frequent dinucleotide was GC/CG (3.81%).

3.3. Frequency and nature of amino acids generated from the trinucleotide repeat motifs

With the advent of bioinformatics, proteins have become increasingly studied at a genomic level. As a result, the practice of representing the genetic code as a DNA codon has become more popular. In the present study, each trinucleotide motif codes an amino acid that has putative roles in the biological activity of protein molecules. Table 5 clearly revealed that out of a total of 63 trinucleotides 11.11% of trinucleotides SSRs encoded isoleucine, which is nonpolar, and in total 47.06% polar and 52.94% nonpolar amino acids were detected.

3.4. BLASTx analysis and functional domain analysis

A total of 163 sequences were judged for BLASTx analysis and, based on this analysis, a putative function was assigned to 134 of the sequences (82.2%). The annotation results indicated that most of the EST-SSR sequences showed high homology with known *Arabidopsis thaliana*, *Helianthus annuus*, and *Medicago truncatula* proteins. Functional domain analysis revealed that most of the EST-SSR sequences found the protein domains responsible for VWFC domain signature, EGF-like domain signature 1,

and 2Fe-2S ferredoxin-type iron-sulfur binding region signature. These regions are generally important for the function of a protein and/or for the maintenance of its three-dimensional structure. Based on the result of the PROSITE ExPASy tool, out of 163, 150 sequences (92.02%) containing protein domains were identified, which are presented in Table 6. A total of 11 polymorphic primers were obtained, while six primers were found to be monomorphic and one primer did not show any amplification.

3.5. PCR amplification

After using the Primer3 software (www.frodo.wi.mit.edu/primer3) 18 primers were custom synthesized from the SSR containing 18 singletons randomly chosen from a total of 3845 singletons and these tested primers generated 61.11% polymorphism. The results indicated that out of all 18 EST-SSRs, 17 primer pairs gave scorable bands, 11 of which were polymorphic and 6 were monomorphic. One EST-SSR did not give any PCR amplification. The alleles produced were varied from single to multiallelic and the amplicon size varied from 100 bp to 1200 bp only. A total of 241 fragments were generated by all 17 primers that showed amplification, i.e. 14.2 fragments per primer.

Jaccard's similarity matrix coefficient obtained through NTSYSpc is presented in Table 7, generated through EST-SSR markers.

Table 7 clearly shows that EST-SSR primer coefficient values ranged from 0.295 to 0.824. The highest value of similarity (0.824) was found between NSt-1 and NSt-3; both were collected from Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan District. The minimum correlation coefficient (0.295) was obtained between St-3 collected from Neri, Research station, Hamirpur, and "A". The average correlation coefficient for EST-SSR obtained was 0.56.

According to the data obtained from Table 8 EST-SSR primer dissimilarity coefficient values ranged from 0.111 to 0.477. The highest value of dissimilarity (0.477) was found between St-3 collected from Neri, Research station, Hamirpur, and "A" collected from Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan District. NSt-1 and NSt-3, both collected from Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan District, were found to have a minimum dissimilarity value of 0.111. The results obtained were in agreement with the results produced by Jaccard's similarity coefficients.

Table 2. Results of EST sequence assembly generated by EGassembler.

Name of crop	Total Number of EST sequences	Number of singletons	Number of contigs	Nonredundant data set (singletons + contigs)	Reduction in redundancy
<i>Stevia</i>	5548	3845	471	4316	22.2%

Table 3. Summary of SSRs found in EST sequences obtained from NCBI.

SSR type	Total number	Frequency
Dinucleotides	105	62.5%
Trinucleotides	63	37.5%
Total	168	

At 38% similarity “A” was singled out from the rest of the 15 collections. Maximum similarity was observed between NSt-1 and NSt-3, both Solan collections of stevia, collected from Dr Y S Parmar UHF, Nauni; and Nst-2, again collected from Dr Y S Parmar UHF, Nauni, was found to be included in the same cluster. Collection Rst-6 (replication cutting of St-6, collected from IARI, New Delhi) was found to be separate from the rest of the 14 collections as

it clustered out in the RAPD and ISSR cluster. In the EST-SSR cluster analysis it was clearly observed that at around 70% similarity St-1, St-2 and Rst-1, Rst-2 formed similar clusters as Rst-1 and Rst-2 were replication cuttings of St-1 and St-2, respectively, collected from Palampur, IHBT, and Dharamshala, from a local farmer, District Kangra (Figure). Polymorphism information content for EST-SSR primers ranged from 0.00 to 0.71 and hence gave an average polymorphism information content of 0.355.

4. Discussion

In recent years, ESTs have become a valuable tool for genome analyses and are currently the most widely used approach for structural and functional plant genomes (Pashley et al., 2006). EST analysis provides a simple strategy for studying the transcribed regions of genomes; before detection of SSRs in ESTs, the ESTs must be trimmed to reduce the redundancy.

Table 4. Distribution of repeat motifs found in EST sequences obtained from NCBI.

S. No.	Repeat Motif	Number	Frequency
Trinucleotide repeats			
	ATC/CAT/TCA	$7 + 4 + 3 = 14$	22.2%
	TAG/ATG/GAT/GTA/TGA	$6 + 2 + 4 + 1 + 1 = 14$	22.2%
	ACC/CCA	$8 + 2 = 10$	15.87%
	GGT/TGG	$1 + 2 = 3$	4.76%
	GAA	6	9.52%
	CCG/GCC	$1 + 1 = 2$	3.17%
	AAC	1	1.58%
	AAT	2	3.17%
	CGG	1	1.58%
	AGA	2	3.17%
	CTT	3	4.76%
	GCA	1	1.58%
	TGC	1	1.58%
	TAT/TTA	$1 + 1 = 2$	3.17%
	CAC	1	1.58%
	Total	63	
Dinucleotide repeats			
1.	GA/AG	$5 + 10 = 15$	14.30%
2.	AC/CA	$10 + 6 = 16$	15.23%
3.	CT/TC	$15 + 19 = 34$	32.40%
4.	TG/GT	$2 + 5 = 7$	6.66%
5.	AT/TA	$22 + 7 = 29$	27.60%
6.	GC/CG	$1 + 3 = 4$	3.81%
	Total	105	

Table 5. List of amino acids and their occurrence frequency depending upon the trinucleotide repeat motifs obtained.

S. no.	Amino acid	Trinucleotide	No. of times repeated	Frequency of occurrence	Nature of amino acid
	Isoleucine	ATC	7	11.11%	Nonpolar
	Histidine	CAT, CAC	4 + 1 = 5	7.93%	Polar
	Serine	TCA	3	4.76%	Polar
	Stop codon	TAG	6	9.52%	-
	Methionine	ATG	2	3.18%	Nonpolar
	Aspartic acid	GAT	4	6.35%	Polar
	Valine	GTA	1	1.59%	Nonpolar
	Stop codon	TGA	1	1.59%	-
	Threonine	ACC	8	12.7%	Polar
	Proline	CCA, CCG	2 + 1 = 3	4.76%	Nonpolar
	Glycine	GGT	1	1.59%	Polar
	Tryptophan	TGG	2	3.17%	Nonpolar
	Glutamic acid	GAA	6	9.53%	Polar
	Alanine	GCC, GCA	1 + 1 = 2	3.17%	Nonpolar
	Asparagine	AAC, AAT	1 + 2 = 3	4.76%	Polar
	Arginine	CGG, AGA	1 + 2 = 3	4.76%	Polar
	Leucine	CTT	3 + 1 = 4	6.35%	Nonpolar
	Cysteine	TGC	1	1.59%	Nonpolar
	Tyrosine	TAT	1	1.59%	Nonpolar

In stevia information on the existence of molecular markers is limited. A few earlier studies have demonstrated the same number of ESTs in stevia as has been reported in the current study. However, none of the studies have reported the detection of SSRs from stevia ESTs. Ours is the first study to generate EST-SSRs and use them for diversity analysis as well as for functional annotations. ESTs, being transcribed regions of the genome, provide a valuable source for candidate gene discovery.

Brandle et al. (2002) and Richman et al. (2005) studied ESTs in stevia and reported that they can be of great help in identification of candidate genes for secondary metabolites specifically for diterpenes, which forms a part of steviol glycoside.

In recent years, in silico approaches have been effectively utilized to detect SSR amplifying genic regions (Igarashi et al., 2008; Woodhead et al., 2008). The development of SSR markers following the conventional approach of genomic library construction is time consuming (Zane et al., 2002) and resource intensive (Squirrell et al., 2003).

Among all the SSR motifs, dinucleotide SSRs were abundantly present. Among trinucleotide repeat motifs the dominant repeat motif was ACC, followed by

ATC. Among dinucleotide repeats the most frequent repeat was CT/TC, followed by AT/TA, and the least frequent dinucleotide was GC/CG. This was found to be in agreement with studies in the traditional Chinese medicinal plant *Epimedium sagittatum* (Zeng et al., 2010) and also in coffee (Aggarwal et al., 2007). Di-tetra or pentanucleotide variation would produce shifts in the reading frame that would result in negative selection and a lower degree of polymorphism (Varshney et al., 2002). Hence, in the present study, trinucleotide repeats in SSRs were given preference. The trinucleotide SSRs are triplet codons that code for a particular amino acid. The triplet codons form an open reading frame (ORF) translated to proteins. Out of a total of 63 trinucleotides, the majority of trinucleotides SSRs encoded isoleucine, which is nonpolar. The analysis of data also revealed that the majority of amino acids were nonpolar.

The annotation results of BLASTx analysis indicated that most of the EST-SSR sequences of *S. rebaudiana* showed high homology with known proteins of *Arabidopsis thaliana*, *Helianthus annuus*, and *Medicago truncatula* proteins, and functional domain analysis revealed that most of the EST-SSR sequences found the protein domains responsible for VWFC domain

Table 6. BLASTx, functional domain analysis, singletons that showed homology to genes with proteins matching *Stevia rebaudiana* identified in a BLASTx search of the NCBI database and SSR markers generated.

S.no.	Sequence Id	Protein	Source	Primer sequence	E-value	Percent similarity	Protein domain	PIC	Polymorphic/ monomorphic and amplicon size range
1.	gi 18465673	hAT-like transposase	<i>Arabidopsis thaliana</i>	F: CGGGTTAGAAAGGAAACGTGA R: AAGTTCCACCAACCCATCA	9e - 59	63%	2Fe - 2S ferredoxin -type iron - sulfur binding region signature	0.71	Polymorphic 500 bp to 800 bp.
2.	gi 18465467	expressed protein	<i>Oryza sativa Japonica</i>	F: CAGGAACACCGATAAATGGAA R: TCAATGGTCAGACAAACACCA	6e - 06	52%	Thiolases active site	0.00	Polymorphic 400 bp to 870 bp.
3.	gi 18465444	Ribosome biogenesis protein BMS1 -like protein	<i>Medicago truncatula</i>	F: ATGAAAGCGAGCCTGATGAT R: TCAAGCAACGATTCCTTTCCA	2E - 26	38%	VWFC domain signature	0.50	Polymorphic 100 bp to 610 bp.
4.	gi 18464838	DnaI domain containing protein, expressed	<i>Oryza sativa subsp. japonica (Rice)</i>	F: CTTTCCGTCAGGAGTTCAGC R: AATGGCAATTCACGAAGAG	1E - 36	50%	VWFC domain signature	0.66	Monomorphic 350 bp to 700 bp.
5.	gi 18464578	Calmodulin-binding protein-like protein	<i>Arabidopsis thaliana</i>	F: CAAAAGAAAGGCTCCCATCAA R: TTTCTGTGGAGTTGCAGGTG	2E - 4	35%	4Fe - 4S ferredoxin-type iron - sulfur binding region signature	0.66	Polymorphic 620 bp and 750bp.
6.	gi 18464496	B2 protein	<i>Daucus carota</i>	F: GGGAAACATGGGAAGAACAA R: CCGGTGTGATTTGCCTTACT	2E - 69	79%	2Fe - 2S ferredoxin-type iron - sulfur binding region signature	0.00	Monomorphic 800 bp to 1100 bp.
7.	gi 16950303	Probable polyprotein allergen Hgg - 14	<i>Heterodera glycines (Soybean cyst nematode worm)</i>	F: GATTCCAATACACGCGCTTT R: CAAAAGCCCCACCAACATTT	1.5 E - 2	80%	4Fe - 4S ferredoxin-type iron - sulfur binding region signature	0.49	Polymorphic 510 bp to 800 bp
8.	gi 16950250	S - adenosylmethionine decarboxylase uORF	<i>Daucus carota</i>	F: TCAAAGTTAGGGTTCCGTTTCG R: GCCGTTTTTTCGTATCCTTCA	5e - 14	100%	2Fe - 2S ferredoxin-type iron-sulfur binding region signature	0.00	Monomorphic 500 bp and 600 bp
9.	gi 16950178	Ribosomal RNA processing protein-like protein	<i>Medicago truncatula</i>	F: TGTAATTTGGGCACAATCG R: GGAACATCTTCATCGCCATT	1e - 23	43%	Thiolases active site	0.54	Polymorphic 500 bp to 750 bp.
10.	gi 16949890	Kinase-related protein	<i>Arabidopsis thaliana</i>	F: GCAGCAAAACCCTAGAGACG R: CTTTCAGGGCACAGAAAAGC	3e - 11	47%	VWFC domain signature	0.00	Monomorphic 520 bp to 700 bp.
11.	gi 16949765	Overexpressor of cationic peroxidase 3	<i>Arabidopsis thaliana</i>	F: CAAGGCTTGCTCCGAAATAC R: TCATCTGCAAGTCTTCCTC	8.8	76%	VWFC domain signature	0.35	Polymorphic 680 bp to 900 bp

Table 7. Jaccard's similarity coefficients of *Stevia rebaudiana* based on EST-SSR markers.

	A	B	St-1	St-2	St-3	St-4	St-5	St-6	NSt-1	NSt-2	NSt-3	CSt-1	RSt-1	RSt-2	RSt-5
B	0.568														
St-1	0.450	0.693													
St-2	0.372	0.612	0.714												
St-3	0.295	0.517	0.576	0.618											
St-4	0.344	0.526	0.642	0.629	0.644										
St-5	0.351	0.547	0.641	0.660	0.642	0.750									
St-6	0.357	0.574	0.666	0.720	0.666	0.709	0.780								
NSt-1	0.396	0.578	0.666	0.625	0.612	0.677	0.649	0.672							
NSt-2	0.360	0.559	0.672	0.690	0.619	0.629	0.627	0.736	0.733						
NSt-3	0.360	0.559	0.672	0.690	0.672	0.711	0.714	0.767	0.824	0.766					
CSt-1	0.415	0.615	0.711	0.700	0.540	0.631	0.660	0.654	0.655	0.719	0.781				
RSt-1	0.389	0.542	0.573	0.586	0.578	0.612	0.583	0.580	0.634	0.615	0.666	0.616			
RSt-2	0.327	0.508	0.568	0.581	0.573	0.557	0.578	0.603	0.580	0.666	0.612	0.586	0.706		
RSt-5	0.381	0.603	0.698	0.653	0.610	0.620	0.711	0.642	0.701	0.677	0.706	0.716	0.661	0.660	
RSt-6	0.395	0.551	0.588	0.571	0.508	0.666	0.538	0.596	0.543	0.578	0.551	0.576	0.618	0.448	0.596

Table 8. Dissimilarity coefficients of EST-SSR analysis generated by DARwin5 ver. 5.0.155.

	A	B	St-1	St-2	St-3	St-4	St-5	St-6	NSt-1	NSt-2	NSt-3	CSt-1	RSt-1	RSt-2	RSt-5
B	0.211														
St-1	0.311	0.166													
St-2	0.355	0.211	0.155												
St-3	0.477	0.311	0.277	0.233											
St-4	0.422	0.300	0.222	0.222	0.233										
St-5	0.388	0.266	0.211	0.188	0.222	0.144									
St-6	0.400	0.255	0.200	0.155	0.211	0.177	0.122								
NSt-1	0.388	0.266	0.211	0.233	0.266	0.211	0.222	0.211							
NSt-2	0.433	0.288	0.211	0.188	0.266	0.255	0.244	0.166	0.177						
NSt-3	0.433	0.288	0.211	0.188	0.222	0.188	0.177	0.144	0.111	0.155					
CSt-1	0.344	0.222	0.166	0.166	0.311	0.233	0.200	0.211	0.222	0.177	0.133				
RSt-1	0.400	0.300	0.288	0.266	0.300	0.266	0.277	0.288	0.255	0.277	0.233	0.255			
RSt-2	0.433	0.311	0.277	0.255	0.288	0.300	0.266	0.255	0.288	0.222	0.266	0.266	0.188		
RSt-5	0.377	0.233	0.177	0.200	0.255	0.244	0.166	0.222	0.188	0.211	0.188	0.166	0.222	0.211	
RSt-6	0.322	0.244	0.233	0.233	0.311	0.188	0.266	0.233	0.288	0.266	0.288	0.244	0.233	0.355	0.233

signature, EGF-like domain signature 1, and 2Fe-2S ferredoxin-type iron-sulfur binding region signature.

The detection of a higher level of polymorphism, i.e. 61.11%, using SSRs was in agreement with reports by some other researchers: Pinto et al. (2004) recorded 68.6% polymorphism in sugarcane and Chen et al. (2006) reported 59.3% polymorphism

in citrus. The high PIC presented would facilitate QTL identification and marker-assisted selection due to the association with functional regions of the genome.

The present study clearly revealed a great level of variation among all the collections. Dendrogram clustering in the present study based on UPGMA

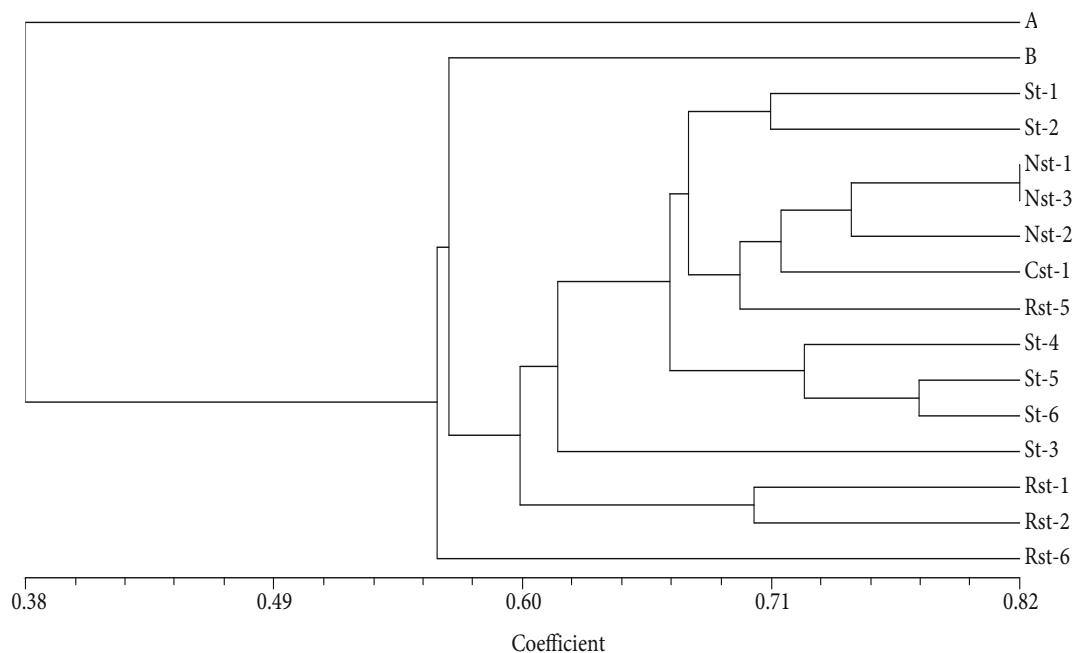


Figure. Dendrogram derived using EST-SSR primers.

grouped various stevia collections based on their response to EST-SSR primers that until now had done based only on their morphology.

The present study is an initiative in the direction of development of EST-based microsatellite markers that

can be used for genetic studies of *Stevia rebaudiana*. It is hoped that with the increasing emphasis on computational biology large EST resources will become available for stevia in the near future for designing various breeding programs.

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