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ONLINE RESOURCES

Functionally relevant novel microsatellite markers for efficient genotyping in *Stevia rebaudiana* Bertoni

ABHISHEK BHANDAWAT[1], HIMANSHU SHARMA[1], AKSHAY NAG[1], SANATSUJAT SINGH[2], PARAMVIR SINGH AHUJA[1] and RAM KUMAR SHARMA[1]*

[1]*Biotechnology Division and* [2]*Floriculture Division, CSIR-Institute of Himalayan Bioresource Technology, P. O. Box No. 6, Palampur 176 061, India*

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Introduction

Stevia rebaudiana Bertoni is a perennial herb that belongs to family Asteraceae. It produces rebaudioside which is one of the key components responsible for extraordinary sweetness of stevia. It produces steviol glycosides which are proved beneficial for diabetes type II patients and controlling blood pressure in hypertension patients (Hsieh *et al.* 2003; Gregersen *et al.* 2004). Moreover, stevia is a rich source of antioxidants, antimicrobial and antifungal compounds (Lemus-Mondaca *et al.* 2012), imparting great economic and commercial value to this crop. Stevia is indigenous to Central and South America (Brandle *et al.* 2002). However, it was introduced in the late 1990s in India. Self-incompatibility, poor-seed viability, low germination rates and entomophilous pollination are among the major challenges in management and genetic improvement of stevia through conventional breeding approaches (Mitra and Pal 2007).

To date, population genetic analysis in *S. rebaudiana* has been performed using arbitrary markers such as RAPD, AFLP and ISSRs (Yao *et al*. 1999; Heikal *et al*. 2008; Hassanen and Khalil 2013) which lead to underestimation of the recessive allele frequency in a population causing a bias in the estimates of genetic diversity and differentiation (Nybom 2004). Further, limitations such as low reproducibility rate, inability to detect heterozygous individual or/and locus non-specificity, makes them less preferred markers (Kalia *et al.* 2011). In contrast, unigene derived microsatellite markers developed from public database, supersede other markers due to their codominant nature, hypervariability, genomewide occurrence, robustness and ability to establish markers–trait association (Provan *et al.* 2001; Chung *et al.* 2006). Nevertheless, such markers are not yet reported in Stevia, limiting its diversity characterization and genetic improvement through breeding. The current study focuses on development of functionally relevant microsatellite markers from publicly available expressed sequence data (ESTs) and evaluation of their potential for diversity characterization of *Stevia* germplasm.

*For correspondence. E-mail: mrk_sharma@yahoo.com, ramsharma@ihbt.res.in.

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Materials and methods

Plant material and DNA extraction

Simple sequence repeat markers were validated in 40 randomly selected genotypes (named as SR-1 to SR-40) of *S. rebaudiana*, maintained at CSIR-IHBT, Palampur, India. Genomic DNA was isolated from fresh leaves of individual genotype using CTAB method as described by Doyle and Doyle (1990) with minor modifications.

Data mining and marker development

A total of 5548 ESTs of *S. rebaudiana* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/entrez) on 3 January 2012 and assembled to potential unigenes using SeqMan DNA Star lasergene ver. 7.1 with default parameters (DNASTAR, Madison, USA). All the predicted unigenes were subsequently searched for the presence of microsatellites (motif lengths 2–6) using Repeat masker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). SSRs were classified as class I (repeat number $\geq$ 20 nucleotides) and class II (repeat numbers 12–19 nucleotides). Primer pairs for microsatellite sequences were designed using Primer3

ver. 0.4.0 (http://frodo.wi.mit.edu/) and prefixed as SUGMS (*Stevia* UniGene derived MicroSatellites) markers.

Amplification profiling

All PCR reactions were performed in a 10 μL reaction volume consisting of 1× PCR buffer (10 mM Tris-pH 9.0, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$), 15 ng each of forward and reverse primers, 200 μM of each dNTPs, 0.2 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 20 ng of template DNA. All the PCR reactions were carried in I-Cycler (Bio-Rad, Berkeley, USA). The PCR protocol consisted of one denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, annealing at optimum temperature (T_a) (table 1) for 1 min, extension at 72°C for 1 min and final extension step at 72°C for 7 min.

PCR amplicons were separated on denaturing polyacrylamide gels consisting of 7% polyacrylamide (acrylamide : bis-acrylamide 19 : 1) and 7 M urea in 1× TBE buffer. The PCR reactions were mixed with half the volume of denaturing dye (98% formamide containing 0.8 mM EDTA and 0.025 % of each bromophenol blue and xylene cyanol), denatured at 94°C for 5 min and snap cooled on ice. Samples along with 50 bp ladder (Fermentas, Vilnius, Lithuania) were loaded on preheated sequencing gel at 55 W for 1.5 h. After run, amplified fragments were visualized using standard silver staining protocol (Promega, Madison, USA).

Functional annotation

Putative functions for SSR containing unigenes were determined using NCBI BLASTx search tool available at TAIR (http://www.arabidopsis.org/Blast/index.jsp).

Data analyses

PCR fragments were scored in the form of binary data, presence (1) or absence (0) of bands and entered in the Excel sheet. Observed and expected heterozygosity was determined using Popgene ver. 1.32 (Yeh *et al.* 1997). Unweighted pair group method with arithmetic mean (UPGMA) analysis was performed to generate a dendrogram based on Jaccard's coefficient using DARwin5: ver. 5.0.158 (Perrier *et al*. 2003).

Results and discussion

A total of 2977 unigenes (2225 singletons and 752 contigs) representing 2.58 Mb of sequence were predicted from 5548 publicly available ESTs of *S. rebaudiana* by clustering of 2–15 random EST sequences. Ninety-nine unigenes containing 107 SSRs (excluding monorepeats) were identified. Microsatellite frequency was found to be one in every 26 Kb of nonredundant EST data, with tri-repeats being the most abundant (72.9%) followed by di-repeats (11.2%), hexa-repeats (6.5%), tetra-repeats (5.6%) and penta-repeats (3.7%). Variation in trinucleotide repeat leads to polymorphism with fewer chances of frame-shift mutation in essential genes. Among tri-repeats, $(ATG/CAT)_n$ type of repeat motifs coding for methionine/histidine were most predominant (43.3%).

Ninety-five (96%) nonredundant SUGMS primer pairs flanking different repeat motifs were identified from SSR containing unigenes. Eighty-five SUGMS markers were highly efficient with amplification success rate of 89.5%, showing that unigene-derived microsatellites are robust source of SSR marker development. Each marker was subsequently utilized evaluating 40 stevia accessions. Fifty-two primer pairs (61.2%) were found to be polymorphic. Number of alleles ranged from 2–15 with an average of 4.7 alleles per SSR locus. Maximum alleles were observed in case of SUGMS 49 (15 alleles). Considering high level of allele frequency and polymorphic potential., these markers could be proved more informative for diversity, genome mapping and screening of elite varieties of stevia. Remaining 33 (38.8%) markers targeting highly conserved regions produced monomorphic amplification pattern.

All the 99 SSR containing unigenes were searched against *Arabidopsis* proteome for putative function. Eighty-seven (87.9%) were found to show significant homology to known genes which suggests promising role of SSRs in functional domain of stevia. In many eukaryotes, repeat length variation is known to play an important role in adaptation to environmental stress by generating favourable alleles through recombination or mutations. Significance of SSRs in adaptability against biotic and abiotic stresses in *S. cerevisiae* and sugarcane has been reported (Parida *et al.* 2010). Ten markers namely, SUGMS 5 (response to salt stress), SUGMS 15 (cadmium stress) SUGMS 17 (biotic and abiotic stress), SUGMS 18 (methyl transferase), SUGMS 21 (response to fungus, cold and drought), SUGMS 22 (drought), SUGMS 35 (osmotic stress and salt tolerance), SUGMS 39 (response to fungus and cold), SUGMS 42 (response to hormones, cadmium and karrikins) and SUGMS 46 (defence), showing substantial polymorphism were identified. Two polymorphic makers namely, SUGMS 28 and SUGMS 43, related to steviol biosynthesis were also characterized. Additionally, 17 markers related to developmental and vegetative to flowering phase transition, and six related to cell cycle regulation were also identified. Details of markers and their role in specific biological process are provided in table 1.

Expected heterozygosity (H_e) and observed heterozygosity (H_o) ranged from 0.03–0.92 (avg. 0.63) and 0.03–1.00 (avg. 0.80), respectively. Heterozygosity values of almost all markers showed deviation ($H_o > H_e$) from Hardy–Weinberg equilibrium (HWE) at $P < 0.05$, therefore, suggesting highly outcross nature of this crop. Genetic diversity based on Jaccard coefficient ranged from 39.2 (between SR-30 and SR-31) to 76.1% (between SR-19 and SR-26) with an overall diversity of 61.3%. Cluster analysis based on 247 alleles derived from 52 polymorphic primers were successfully tested for diversity characterization of 40

Table 1. Characteristics and amplification potential of 52 SUGMS markers in 40 genotypes of *Stevia rebaudiana*.

Locus ID	Constituting ESTs	Primer sequence (5′–3′)	T_a (°C)	Repeat motif	Function, accession ID of TAIR	No. of alleles	H_o	H_e	Product size (bp) Expected	Observed
SUGMS 1	BG525321	F: TCCTCATCATCATCATCACAAA R: GGTAAGTGGATCATCAAGTCTGA	55	$(CAT)_{32}$	Exostosin family protein, AT5G37000	6	1.00	0.67	219	300–420
SUGMS 2	BG521424, BG525530, BG523655, BG522604, BG526690	F: CACAATGGCAGCAATAACCA R: GCCATCATCGTCACTCCTCT	55	$(CCA)_{18}$	Protein kinase superfamily protein, AT1G13350	2	0.72	0.61	150	140–180
SUGMS 3	BG524717, BG524235, BG526288, BG526657, BG522121, BG526332, BG522520, BG526416, BG521510	F: TTTTGGTAGAGCCATCACCAC R: TTACACATTGGAAGCCAGCA	55	$(ATGGTG)_{10}$	Ribosomal L29 family protein, AT5G02610	2	0.76	0.71	228	400–500
SUGMS 4	BG525563	F: CACAGCTGGCACAAAAAGAC R: CTTTGTGGCTGGTTTTCTGG	55	$(TTTTA)_4$	Unknown protein, AT4G27610	4	0.86	0.72	210	200–310
SUGMS 5	BG522142, BG525446, BG525475	F: TCTCTGCACCATGACTGCTC R: GATCTCCCACTTGCATCTCC	55	$(ATG)_{19}$	Nascent polypeptide-associated complex subunit alpha-like protein 3 (NACA3), AT5G13850	3	0.80	0.55	177	143–152
SUGMS 6	BG522380	F: ATGAAAGCGAGCCTGATGAT R: TCAAGCAACGATTCTTTCCA	55	$(GAA)_{11}$	Protein kinase superfamily protein, AT5G44290	6	1.00	0.77	187	450–555
SUGMS 7	BG525676	F: CAATTTAACTGCCACGAGAGG R: AGGAGGCCGATATGTCAAAA	55	$(TTA)_6$	Transposable element gene, AT4G04120	2	1.00	0.51	153	120–130
SUGMS 8	BG525676	F: CGGCCTCCTACAAACCCTAT R: GTTTGTTCTTCGCGGTTGAT	55	$(ATG)_{10}$	Transposable element gene, AT4G04120	3	1.00	0.62	225	250–300
SUGMS 9	BG522915	F: CAACTGAACCAGAAGCCAAAG R: CATGGTCCTCATCTTCATCC	55	$(ATG)_{59}$	Unknown protein, AT1G76070	3	0.80	0.75	230	260
SUGMS 10	BG525252	F: TGCTCACCGAAACCCTAATC R: CTCCACCCGGTGTCTCTTTA	55	$(CCA)_8$	Unknown protein, AT3G52740	6	0.67	0.60	247	250–335
SUGMS 11	BG522215, BG525813	F: TCCCAAGGCTAGAACTCGTG R: GCACTCGCCCTGTTTGTATT	55	$(CAT)_9$	DNAse I-like superfamily protein, AT5G04980	3	0.54	0.68	204	200–250
SUGMS 12	BG524668	F: GTCGGTTGGTTCTCACAACA R: TCTTTTCACCCATCCTGCTC	55	$(CAT)_{10}$	Metal-dependent phosphohydrolase, AT1G26160.1	2	0.18	0.16	224	410–412
SUGMS 13	BG523875	F: GTAGTAGCAGTGGCGGTGGT R: CCGGGTTATCCTGGTTAGGT	55	$(ATG)_{26}$	encodes a plasmodesmal (Pd)-associated membrane protein, AT5G42100.2	8	0.76	0.71	201	225–290
SUGMS 14	BG522917	F: CCCTTCTATCTTCATCCCACA R: CCCTTCTATCTTCATCCCACA	55	$(CCA)_{10}$	Encodes subunit NDH-N of NAD(P)H, AT5G58260.2	5	0.96	0.76	250	280–350
SUGMS 15	BG524407, BG522834	F: CTCAAACCATTGCAGCTGAT R: TGCCAAACTTGCTGAGAAGA	55	$(CAG)_{30}$	Encodes one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins, AT4G22670.1	2	0.03	0.03	249	570–580
SUGMS 16	BG525285, BG524211	F: ATGCCAAAAGGAGTTGTCGT R: CAGAAGAGAGGGAGCCTAGC	55	$(TTA)_{12}$	squamosa-promoter binding protein-like, AT3G15270.1	3	0.44	0.37	204	265–275
SUGMS 17	BG521339, BG524567, BG521545	F: ACCTGACGCATGATAGGAAA R: GTGACCGCGTCTCTCAAGAT	55	$(TAA)_8$	ATBETAFRUCT4, AT1G12240.1	2	0.38	0.31	178	190–198

Table 1 (*contd*)

Locus ID	Constituting ESTs	Primer sequence (5′–3′)	T_a (°C)	Repeat motif	Function, accession ID of TAIR	No. of alleles	H_o	H_e	Product size (bp) Expected	Observed
SUGMS 18	BG523972	F: GGAAAGAATGCCGAATTTGA R: TGAGGATGAAGACGATGCTG	55	$(TAA)_{11}$	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein, AT1G29790.2	4	0.76	0.66	169	200–230
SUGMS 19	BG521353	F: AGATAACCGGGAAACCTTCG R: GTACGCCACCGGATTGTAGT	49	$(CCA)_6$	Unknown protein, AT2G33850.1	5	1.00	0.76	163	200–238
SUGMS 20	BG522115	F: ACAGCAAACAAGCAAGCAAA R: CGGGTCACAAGTTGGTGTTT	49	$(CCA)_{10}$	D6PK is a protein kinase, AT5G55910.1	2	0.22	0.20	208	200–230
SUGMS 21	BG521793	F: ACCCACAAGGATGCTCAATC R: TATCCCGGCCTTCCTTTATT	55	$(TTTTA)_5$	Heavy metal transport/detoxification superfamily protein, AT1G51090.1	3	0.77	0.63	175	200–220
SUGMS 22	BG524176	F: CCAAAACCCGCTTAGTGAGA R: TGCTTTAAAAACCAACCGAAA	55	$(TAA)_{12}$	Drought-responsive family protein, AT4G02200.3	6	0.99	0.83	187	195–225
SUGMS 23	BG523553	F: CGCGATTGCTTTGAAATATG R: CTTCCTCCTACGGGATCGAC	55	$(ATG)_{14}$	CAX-interacting protein (CXIP4), AT2G28910.1	4	0.86	0.72	244	250–350
SUGMS 24	BG525470	F: ACCACAACCACCACAAAAGG R: CGGGAGGATGAGAGAGAGTG	55	$(CAT)_{11}$	Ribosomal protein L34e superfamily protein, AT5G19025.1	14	1.00	0.92	209	240–315
SUGMS 25	BG522826	F: AAGGGTGAAAAGCTCGAACA R: TCGGAAAGATTGGATTCTGC	55	$(GAA)_{13}$	Stress response suppressor 1 (STRS1), AT1G31970.1	4	1.00	0.51	242	270–302
SUGMS 26	BG525190	F: TGGTTTTTGACAACGTGGAG R: CCTCCACTTTTCGCCTTTTT	55	$(CAT)_{23}$	Transposable element gene, AT4G07725.1	8	1.00	0.85	247	248–350
SUGMS 27	BG522949, BG523751	F: CGAGTGCAGATCAATTACTGAAT R: GAGGCACTGGGAAGCAATAG	55	$(CAT)_6$	Synaptobrevin-like protein family, AT2G33120.1	4	1.00	0.65	150	147–180
SUGMS 28	BG523058	F: CAAATTGGGAATTGCAGCTT R: GACAAACAAGCCGAGAGAGG	55	$(CAT)_9$	Thylakoid lumenal 17.4 kDa protein, chloroplast, identical to SP:P81760 Thylakoid lumenal 17.4 kDa protein, AT5G53490.3	11	0.98	0.78	208	210–310
SUGMS 29	BG521371	F: GGAAACGTCGGGAACTTGTA R: TTATCACCCCAAGATGCACA	55	$(ATG)_8$	Transducin family protein / WD-40 repeat family protein, AT2G19540.1	3	0.52	0.48	214	360–450
SUGMS 30	BG526290	F: TTCAATCTCTAGCCGGCATC R: GGACGAATCAAACCAAACCA	49	$(CAT)_{16}$	Leucine-rich repeat serine/threonine protein kinase, AT5G46330.1	2	0.32	0.47	170	180–220
SUGMS 31	BG521417	F: CCAACAAGCACCCAATTACA R: GCGGAGATGGTCTCTGTCTC	48	$(TA)_{13}$	Unknown protein, AT1G52140.1	9	0.97	0.82	193	245–352
SUGMS 32	BG523237, BG526314, BG525816	F: AACGCGGTGTATCAGAAAGG R: GTCTCTGCAGCAGGCAAAGT	55	$(CAA)_{18}$	DCD (development and cell death) domain protein, AT3G27090.1	4	0.99	0.73	189	198–220
SUGMS 33	BG523762	F: CGTTTCCCCATTTACCCTTT R: TGCTGCTGCATCATCTTTGT	55	$(TA)_{18}$	Encodes a protein with similarity to mammalian transcriptional coactivator, AT5G28640.1	9	0.94	0.77	193	190–240
SUGMS 34	BG521609	F: TGTCATCAACTTTGATTATCTG R: TTTGCCAACCAATTAGTCCA	55	$(TA)_{12}$	Ribosomal protein L7/L12, oligomerisation Ribosomal protein L7/L12, C-terminal/adaptor protein ClpS-like, AT1G70190.1	3	0.91	0.59	235	250–275
SUGMS 35	BG525396	F: GGAATCGGTGCAGCTATGTT R: TGGAAAGATTTACAATTCACA	55	$(TA)_{23}$	Hexokinase-like 1 (HKL1), AT1G50460.1	3	0.52	0.41	166	196–200
SUGMS 36	BG524287	F: GCTTAGGAAACATGGCGTCT R: GGTCCAAACGCTTTCTCATC	49	$(CGG)_{13}$	Actin binding, AT5G07740.1	6	1.00	0.80	162	160–200

Table 1 (*contd*)

Locus ID	Constituting ESTs	Primer sequence (5′–3′)	T_a (°C)	Repeat motif	Function, accession ID of TAIR	No. of alleles	H_o	H_e	Product size (bp)	
									Expected	Observed
SUGMS 37	BG526856	F: TCATCAAAAGTCGAACCATCA R: CCAAGGGTCGTTTCATCTTT	55	$(CCA)_{17}$	Ubiquitin-protein ligase, AT5G02880.1	3	0.98	0.53	221	237–243
SUGMS 38	BG524986	F: CGTTCGTCCCTCCATCAAT R: GCCCATCCGGTTATCTTTCT	49	$(CA)_{12}$	Encodes a catalytically active cinnamyl alcohol dehydrogenase, AT3G19450.1	6	0.98	0.78	159	175–240
SUGMS 39	BG521432	F: CTTTCTTTTGGCGGGAAAC R: GGGGGTAAACCAAAAAGCTC	55	$(TAA)_{23}$	DCD domain protein, AT5G42050.1	2	0.24	0.22	221	254–258
SUGMS 40	BG524583	F: TTGCAAAACGCAAATCTGAC R: GCGTTTGCCCTTCATTTC	55	$(ATGGTG)_6$	A paternally expressed imprinted gene, AT5G63740.1	7	1.00	0.50	173	170–195
SUGMS 41	BG526779	F: GCACAAAGTCCAGGAGAAGG R: CGAAAATGGGTATAAACCCTACC	55	$(TA)_{12}$	ATP-dependent caseinolytic (Clp) protease/ crotonase family protein, AT4G13360.1	5	0.99	0.78	170	180–195
SUGMS 42	BG524354	F: AGTCAAGGTTGATCCGATGC R: GTCCATGGAATCCCTCGTTT	55	$(TAA)_{11}$	myb-like transcription factor family protein, AT1G70000.2	4	0.97	0.68	231	442–480
SUGMS 43	BG526206	F: CCAATCTACAATTGCCACAAGA R: TTTTCCGAGGTTTTTGGTTG	55	(TTTG)5	Encodes a second Chl I gene (CHLI2), AT5G45930.1	4	0.97	0.73	207	225–255
SUGMS 44	BG521338	F: AAAACCCCAACACAAAGCAC R: CTAGGTTTGTTTCGGGTCCA	55	$(GAA)_{10}$	Encodes Adherin SCC2, AT5G15540.2	3	0.22	0.57	191	200–215
SUGMS 45	BG521658	F: TCCCACTACAGGACCACCTC R: TTCCTGAATTGCAATGGTTG	55	$(CAG)_{10}$	RNA-binding protein 47A (RBP47A), AT1G49600	5	1.00	0.77	193	290–390
SUGMS 46	BG526390	F: AAGCAGTCTATTCAAAAGCCTCA R: CAACAGCAACCTCCAAATGA	55	$(CAAAAA)_3$	Disease resistance protein (TIR-NBS-LRR class) family, AT1G63740.1	6	1.00	0.71	174	172–198
SUGMS 47	BG523473	F: GCAGAAGGGGAAACAATCAA R: GGTAATACGGGGGATGAGGT	55	$(CCCTAA)_4$	Protein of unknown function (DUF295), AT5G55440.1	4	1.00	0.72	168	192–200
SUGMS 48	BG525913	F: GCTAGAAGGCCACCTGGTTA R: CCAGGTTCACCACACTCGTA	55	$(TGG)_{17}$	SR34/SR1 is a plant homologue of the human general/alternative splicing factor SF2/ASF, AT1G02840.3	4	1.00	0.72	215	560–570
SUGMS 49	BG524028	F: GCTGAAAGCCGTTTGAGATT R: CAAACCAACCATCATTAGTCTTTTT	55	$(TTTG)_9$	Unknown protein, AT4G22560.1	15	0.99	0.81	217	200–300
SUGMS 50	BG526327	F: ACCAAAGATCGCCACCAC R: CAAGGCTGAATCGGAAGAAG	55	$(CCA)_{44}$	DEK domain-containing chromatin associated protein, AT5G55660.1	2	0.91	0.59	236	230–250
SUGMS 51	BG524479	F: AATTGATGCTTGCGTTGATG R: CAGGGGGCACTATGGTAAAA	55	$(CATA)_8$	Transposable element gene, AT4G03730.1	8	1.00	0.86	157	175–200
SUGMS 52	BG523678	F: TCCCAATTCAAATCCCTCAA R: CGTTTGTGGTGCAGATTACG	55	$(CCCA)_{22}$	Zinc ion binding, AT5G48205.1	3	1.00	0.59	172	194–200

T_a, annealing temperature; H_e, expected heterozygosity, H_o, observed heterozygosity.

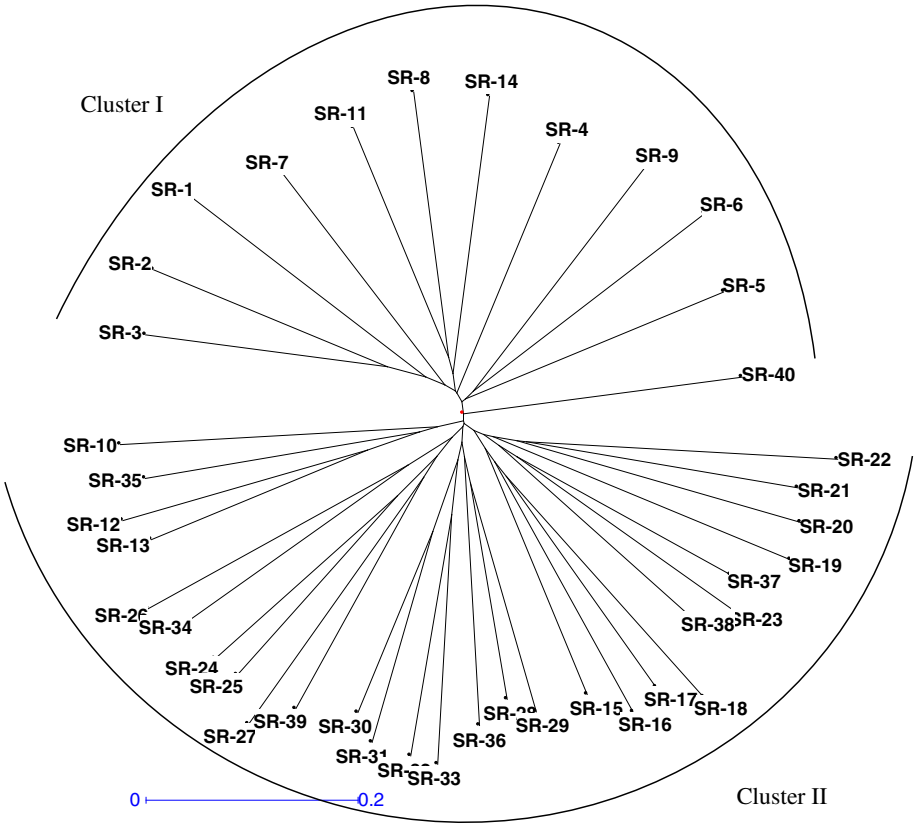

Figure 1. Dendrogram showing genetic relationships among 40 random genotypes of *S. rebaudiana* based on 247 polymorphic alleles produced by 52 SUGMS markers. Scale represents Jaccard's similarity coefficient.

random *S. rebaudiana* genotypes and grouped in two major clusters (figure 1).

In this study, publicly available expressed data in *S. rebaudiana* was successfully used to develop novel genic microsatellite markers. To our knowledge, this is the first set of functionally relevant SSR markers *S. rebaudiana* that would add to the repertoire of unique EST-SSR markers available in Asteraceae. Further, hypervariability potential of novel microsatellite markers can be extrapolated in future genetic diversity and genetic mapping studies in *Stevia*.

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