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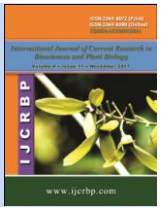

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Assessment of Genetic and Chemo-Diversity among Different Indian Ecotypes of *Stevia rebaudiana* (Bertoni)

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Abstract

The genetic polymorphism and chemo-typing of seven ecotypes of *Stevia rebaudiana* Bertoni collected from different geographical regions of India was investigated by RAPD and LC-MS analysis. Fifty random decamer primers were screened to amplify the genomic DNA from different accessions to check the genetic polymorphism. Of these, eighteen primers selected to generate RAPD finger printing revealed 117 bands, of which 78 (66.67%) were polymorphic. Dendrogram and cluster analysis were constructed based on the unweighted pair group method with arithmetic means (UPGMA). LC-MS based chemo-typing revealed high diversity in the stevioside content in the germplasm/ ecotypes. Data revealed that stevioside content varied greatly from 486 to 986 µg/g of tissue dry weight among the ecotypes with higher content in Himachal Pradesh(SR-HP) followed by Maharastra (SR-MH), Uttar Pradesh SR-UP), Andhra Pradesh (SR-AP now Telangana State), Jammu & Kashmir (SR-JK), Punjab (SR-PB) and Madhya Pradesh (SR-MP). Seven ecotypes were clustered into two major groups and stevioside content variation was linearly associated with these groups. The results suggested that there was a good correlation and association with the data obtained from genetic diversity and phytochemical analysis. Such bioprospecting based studies could be useful to select for superior ecotypes of *Stevia rebaudiana*.

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Keywords

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Introduction

Plants serve as the main source of food and medicine for human and animals. Research efforts in bioprospecting have emerged as an important activity that explores into genetic and chemical diversity of plant species (Ramesha et al., 2011; De Luca et al., 2012). *Stevia rebaudiana* (Bertoni) is an herbaceous perennial shrub belonging to family Asteraceae. *Stevia* has attracted economic and scientific interest worldwide. It is now cultivated in different countries of world, viz. Brazil, Japan, China, Canada, India and Southern Asia, because the leaves of stevia contains zero caloric sweet diterpenoids glycosides. Main steviol glycosides are

stevioside and rebaudioside-A, which are 250-400 time sweeter than sucrose (Goyal et al., 2010). Besides having zero caloric sweeteners, the plant offers several therapeutic benefits such as gluco-regulatory, anti-hypertensive, anti-hyperlipidemic, anticancer, anti-inflammatory, antioxidant and also protects pulmonary and renal function (Gupta et al., 2013).

Stevioside content normally varies from 4-20% in the leaf dry matter, which primarily depends on cultivar and agriculture practices (Geuns, 2000). Dry leaves of stevia are approximately 10 to 15 times sweeter than sucrose (Raymond, 2010) while maintaining glycemic index as zero, (Kroyer, 2010; Tiwari, 2010; Puri et al., 2011) and non toxic effects on human health (Barriocanal et al., 2008). Different biotechnological approaches have been employed for *in vitro* propagation, biomass production and enhanced steviol glycosides production (Modi et al., 2016; Aman et al., 2013).

DNA based markers are successfully used for the identification of genetic variability at intra and inter species level (Naderi et al., 2009). Random amplification of polymorphic DNA (RAPD) technique offers as a fast PCR based molecular method for providing information from large number of loci. RAPD is widely used for various application in plant research and has proved to be a valuable tool in studying inter and intra specific genetic variation, patterns of gene expression and identification of specific genes (Kuddus et al., 2002). RAPD has been used for estimation of plant genetic diversity in *Dendrobium* species (Zha et al., 2009), *Zygophyllum* populations (Hammad and Qari, 2010), *Withania somnifera* (Dharmar and Britto, 2011) and *Cardiospermum halicacabum* (Sheeba et al., 2014).

More recently, RAPD has also been used for identification of intra species genetic diversity among various plant species such as *Musa* (Kiran et al., 2015), *Arachis hypogeal* L. (Al-Saghir and Abdel- Salam, 2015), *Trichosanthes anguina* L. (Rashid et al., 2016) and *Mangifera indica* L. (Pruthvish and Chikkaswamy, 2016) collected from different geographical regions. Chester et al. (2013) conducted RAPD and HPTLC analysis of eleven *Stevia* ecotypes and found much relatedness with each other and superiority of some genotype.

Thiyagarajan and Venkatachalam (2015) reported that the genomic DNA polymorphism and phytochemical variation of *Stevia rebaudiana* (Bertoni) by RAPD-PCR of only three accessions of *S. rebaudiana* (L1 to L3). However it is necessary to screen and identify superior ecotypes for varied applications including biomass, secondary metabolite production or plant improvement. In the present study, we have analyzed the variation at morphological and molecular (RAPD) levels and chemodiversity analysis using LC-MS among seven ecotypes collected from different States of India.

Materials and methods

Plant material collection

In the year 2012, germplasm (hereafter referred to as ecotypes) of *Stevia rebaudiana* was collected in form of plantlets from seven different geographical locations of different states of India, which include Jammu (Jammu and Kashmir), Patiala (Punjab), Palampur (Himachal Pradesh), Indore (Madhya Pradesh), Kanpur (Uttar Pradesh), Hyderabad (Andhra Pradesh), and Pune (Maharashtra). Table 1 present the details about the collection site of different ecotypes with reference to their latitude and longitude. The collected plants were maintained in pots under laboratory conditions viz. 25±2ºC temperature and 12 hrs photoperiod under fluorescent light (1000 -1200 Lux).

Table 1. List of *Stevia rebaudiana* ecotypes and details of their collections sites.

S. No.	State and site of collection	Abbreviations	Latitude*	Longitude*
1	Madhya Pradesh (Indore)	SR-MP	23˚ 30’ N	80˚ 00’ E
2	Uttar Pradesh (Kanpur)	SR-UP	27˚ 40’ N	80˚ 00’ E
3	Himachal Pradesh (Palampur)	SR-HP	32˚ 07’ N	76˚ 32’ E
4	Andhra Pradesh (Hyderabad)	SR-AP	17˚ 22’ N	78˚ 28’ E
5	Maharashtra (Pune)	SR-MH	18˚ 32’ N	73˚ 52’ E
6	Jammu and Kashmir (Jammu)	SR-JK	32˚ 44’ N	74˚ 54’ E
7	Punjab (Patiala)	SR-PB	30˚ 19’ N	76˚ 24’ E

*Source: http: //www.latlong.net/category/cities-102-15.html

Variation at morphological level

Germplasm of *Stevia rebaudiana* collected from different geographical locations (ecotypes) were checked for morphological differences. Three morphological characters viz. leaf morphology (length/ width ratio), pattern of branching (bushy/ spreading) and leaf density were recorded. Data was recorded from approximately 3 months old plants, just before flowering. Leaf length and width were recorded from leaves at 3rd to 5th node. The data was recorded from 5 randomly selected plants and mean was calculated.

Variation at biochemical level

Different ecotypes were analyzed by LC-MS method (Montoro et al., 2013). The crude extract of dried leaves was prepared with HPLC grade methanol using soxhlet apparatus and extract was dried by vacuum evaporator for further use in HPLC- Mass spectrometry. For LC-MS, acetonitrile and water (1:1 v/v) was used as mobile phase and samples were further dissolved in HPLC grade methanol. LC-MS was carried out in a triple quadrupole LC-MS spectrometer in which LC was carried out in an Agilent 1260 infinity equipped with a quaternary pump, online degasser, column heater and autosampler. A Chromolith High Resolution RP-$_{18e}$ column triple quadrupole and MS was in mass spectrometer 6410 with an electrospray ionization source using the facility at the CSIR Lab, IIIM, Jammu.

Genomic DNA isolation

DNA was isolated from leaf samples of *Stevia rebaudiana* from different ecotypes by using CTAB method as described by Doyle and Doyle (1987) with some modifications. DNA was isolated by using 500 mg fresh leaf crushed into fine powder and homogenized in 5 ml extraction buffer (2% w/v CTAB, 100 mM Tris HCl (pH 8), 100 mM Na_2-EDTA, 1.4 M NaCl, 0.2% β mercaptoethanol). The leaf homogenate was incubated at 65 ºC for 90 min then equal volume of chloroform: isoamyl alcohol (24:1 v/v) solution was added and centrifuged at 10,000 rpm for 15 minutes at 4 ºC temperature. The upper layer was separated and mixed with equal volume of isopropanol and incubated overnight at 4 ºC, after that precipitate was washed with 70 % ethanol and air dried. Air dried precipitate was further dissolved in TE buffer and 30 µl RNase was mixed and incubated at 37 ºC for 60 min. Finally product was re-precipitated and dissolved in TE buffer and after quantification used for RAPD analysis.

RAPD Analysis

Fifty random decamer RAPD primers obtained from Operon Technologies Inc. were used for screening. The primers which showed amplification were selected to study genetic diversity using RAPD analysis. PCR was carried out in 20 µl reaction volume containing 2.0 µl 10X PCR Buffer, 2 µl 25 µM dNTP, 1.5 µl 25 mM $MgCl_2$, 0.2 µl 5U/µl Taq DNA polymerase, 2 µl 10 µM/µl primer and 50 ng DNA template.

Amplification reaction was carried out in Eppendorf Master-cycler with an initial denaturation step at 94 ˚C for 5 min followed by 40 cycles of denaturation at 94 ˚C for 1 min, annealing at 36 ˚C for 1min, extension at 72 ˚C for 2 min and final extension at 72 ˚C for 10 min. The RAPD products were loaded with 6X bromo-phenol blue-xylene cyanol DNA loading dye on 1.5 % agarose gel prepared in 1X TE buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at a constant current 60 V for 2 hrs. The size of amplified fragment was determined by using 1 kb ladder (New England Biolabs). Visualization and photography of gel was done with Gel Documentation System (Genetix).

Data analysis

Each gel was analysed by scoring the presence of band as 1 and absence of band as 0. The phylogenetic tree was constructed by using NTSYSpc software version 2.0. Dendrogram was constructed based on UPGMA using similarity between individuals.

Results

Morphological variation

Stevia plants collected from seven different geographical locations differed significantly for morphological attributes such as difference in leaf length: width ratio, branching pattern and leaf density (Fig. 1 and Table 2). The observed variation could not have been due to any other factor such as soil types, amount of rainfall, environmental or climatic conditions, this is because collected ecotypes were allowed to grow under the same environmental and climatic conditions throughout the period of the experiment. There were visual indications of leaf size variations as shown in Fig. 1, but significant

differences were not observed as leaf length and width tends to change equally and proportionately. Therefore the ratio of length/ width remained to be same or constant (Table 2). Three of the ecotypes (SR-MP, SR-UP and SR-MH) exhibited bushy phenotype while SR-HP, SR-AP, SR-JK and SR-PB showed the spreading branching pattern. Leaf density data showed that plant type with bushy phenotype had higher leaf density while the others with spreading types had lower leaf density.

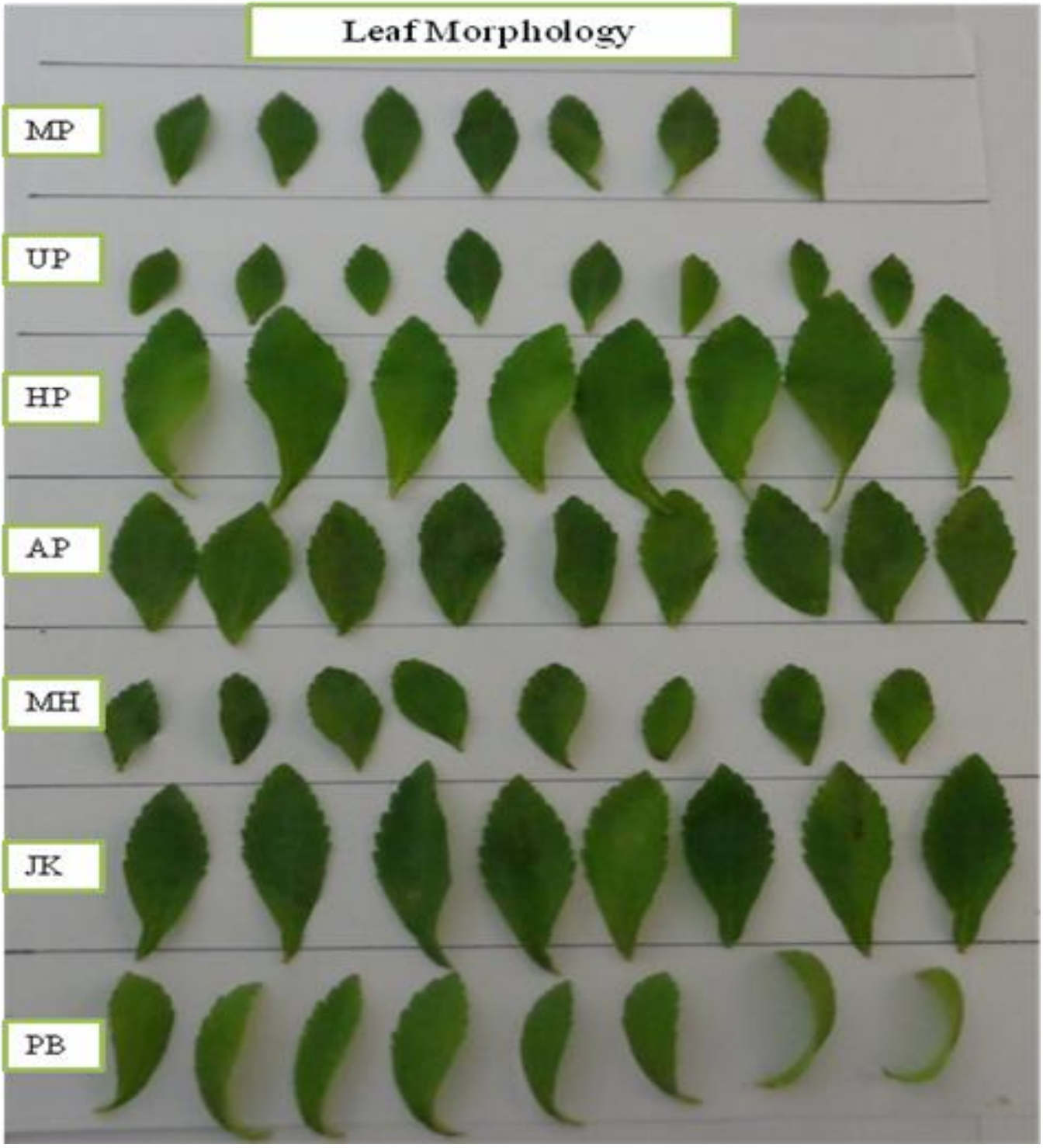

Fig. 1: Variation in leaf morphology among the collected germplasm of Stevia.

Table 2. Different morphological features of collected ecotypes of *S. rebaudiana.*

Sr. No.	Germplasm	Leaf length/width ratio	Branching pattern (bushy/ spreaded)	Leaf density (no. of leaves of 3 month old plant)
1	SR-MP	2.64	Bushy	64±8
2	SR-UP	2.35	Bushy	76±10
3	SR-HP	2.81	Spreaded	56±7
4	SR-AP	2.30	Spreaded	32±5
5	SR-MH	2.48	Bushy	82±6
6	SR-JK	2.92	Spreaded	42±5
7	SR-PB	3.23	Spreaded	28±6

Chemodiversity based on LC-MS analysis

The steviol glycosides content in the leaves of *Stevia rebaudiana* ecotypes was used as biochemical parameter to analyze variation among the ecotypes collected. Standard stevioside and rebaudioside-A were used for the calibration and, stevioside and rebaudioside-A contents in the leaf samples were analyzed. Chromatograms of both standards are given in the Fig. 2. The LC–MS analysis of standard stevioside and rebaudioside-A indicated that retention time of investigated compounds stevioside and rebaudioside- A were 14.2 and 14.3 min, respectively (Fig. 2). The relative concentration of stevioside and rebaudioside- A content of *S.rebaudiana* was quantified by LC-MS analysis (Table 3). The stevioside and rebaudioside- A levels differed considerably among the different ecotypes of the *S. rebaudiana*. The amount of stevioside varied from 486.5±35 µg/g of tissue dry weight (gdw) in SR-MP to 986.8±67 µg/g of tissue dry weight in SR-HP ecotypes and Rebaudioside A varied from 175.3±31 to 268.3±44µg/gdw. Among the collected ecotypes Himachal Pradesh (SR-HP) ecotype found to contain significantly high levels of stevioside (986.8±67 µg/gdw) followed by Maharashtra (704.5±55 µg/gdw), SR-UP (669.9±46 µg/gdw) and SR-AP (648.5±38 µg/gdw). Least amount (486.5±35 µg/gdw) was recorded in SR-MP ecotype (Table 3).

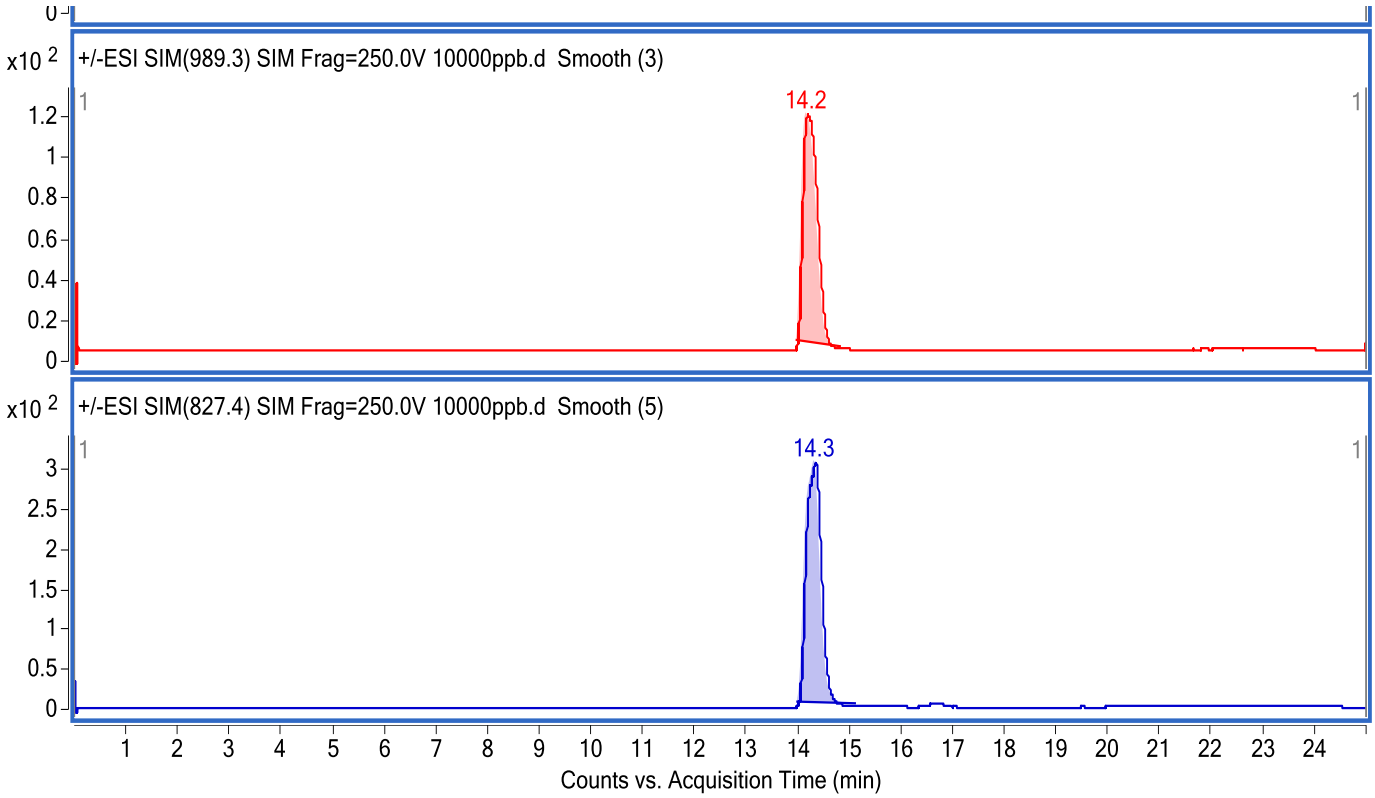

Fig. 2: HPLC chromatogram of standard Stevioside and Rebaudioside-A showing the elution time.

Table 3. Stevioside and rebaudioside- A content (µg/g of tissue dry weight) in different germplasm ecotypes of *S. rebaudiana*.

Sr. No.	Germplasm	Stevioside content	Rebaudioside-A content
1	SR-MP	486.5±35.1	268.3±44.9
2	SR-UP	669.9±46.6	211.3±31.3
3	SR-HP	986.8±67.0	268.6±47.3
4	SR-AP	648.5±38.8	183.8±20.2
5	SR-MH	704.5±55.6	215.1±41.1
6	SR-JK	605.8±63.7	175.3±31.1
7	SR-PB	554.7±24.0	226.5±35.0

Mean value ± standard error value.

RAPD based analysis of genetic variation

All the seven *S. rebaudiana* germplasm ecotypes were analysed using eighteen RAPD primers. The details of primer sequence of primers used to generate RAPD bands, total number of monomorphic and polymorphic bands generated and percentage of polymorphism are summarized in Table 4. Total 117 bands were generated by the eighteen primers tested, in which, 78 (66.67%) bands were polymorphic and 39 (33.33%) bands were monomorphic with average number of polymorphic bands per primer was 4.33. The highest number of bands were observed to be 12 with primer OPX-20 while lowest 4 bands were observed with primer OPAF-02, OPAH-08, OPE-04 and OPZ-01. 100% polymorphism was observed with OPA-08 and OPE-03 primer and 100% monomorphism was observed with primer OPAF-02. The present results thus indicated differential banding pattern and considerable polymorphism among the germplasm ecotypes (Fig. 3). The values derived from the Table 4 showed genetic relatedness among all the seven *Stevia* ecotypes. The resulting dendrogram is shown in Fig. 4; the ecotypes were grouped into two major clusters; one cluster contained only one genotype SR-MP with lower stevioside content but has higher Rebaudioside-A content (Table 3), remaining six ecotypes were grouped into another cluster. Ecotype collected from UP and AP showed highest similarity, while that collected from MP and JK showed highest dissimilarity based on data of similarity coefficients using cluster analysis NTSYSpc Program version (2.0).

Table 4. List of RAPD primers and number of PCR bands amplified from *Stevia rebaudiana* germplasm.

S. No.	Primer	Sequence (5'→ 3')	Total No. of bands	No. of Polymorphic bands	No. of Monomorphic bands	% Polymorphism
1	OPA-03	AGT CAG CCA C	7	5	2	71.42
2	OPA-08	GTG ACG TAG G	5	5	0	100.00
3	OPAB-01	CCG TCG GTA G	6	3	3	50.00
4	OPAB-05	CCC GAA GCG A	6	4	2	66.66
5	OPAF-02	CAG CCG AGA A	4	0	4	0
6	OPAH-08	TTC CCG TGC C	4	3	1	75.00
7	OPC-07	GTC CCG ACG A	8	4	4	50.00
8	OPD-13	GGGGTG ACG A	5	4	1	80.00
9	OPE-03	CCA GAT GCA C	9	9	0	100.00
10	OPE-04	GTG ACA TGC C	4	1	3	25.00
11	OPE-06	AAG ACC CCT C	9	7	2	77.77
12	OPE-08	TCA CCA CGG T	7	5	2	71.42
13	OPG-07	GAA CCT GCG G	7	4	3	57.14
14	OPH-13	GAC GCC ACA C	5	1	4	20.00
15	OPX-20	CCC AGC TAG A	12	10	2	83.33
16	OPZ-01	TCT GTG CCA C	4	2	2	50.00
17	OPZ-04	AGG CTG TGC T	9	6	3	66.66
18	UBC-007	CCT GGG GGT T	6	5	1	83.33
		Total	117	78	39	

Discussion

The extent of genetic variation is of extreme significance in plant improvement programmes aimed at widening the genetic base. *Stevia rebaudiana* is an important crop for the production of non-nutritive, nontoxic, high-potency sweeteners. Understanding the genetic diversity and the production of its basis of glycosides will aid in efforts towards its improvement (Yadav et al., 2011). The limited breeding efforts undertaken to date have not significantly reduced levels of genetic diversity among the stevia breeding lines (Yadav et al., 2011) which could be possible as stevia has not undergone a great deal of selection (Yao et al. 1999). Development of new varieties of *S. rebaudiana* with a higher content of rebaudioside-A and a reduced content of stevioside is the primary aim of plant breeders concerned with the improvement and utilization of this plant containing unique property of natural sweeteners.. In the present study, we have collected different ecotypes of *Stevia* and studied genetic as well as chemo-diversity using molecular and metabolite profiles. Abdullateef and Osman (2011) analyzed 10 stevia accessions collected from different geographical locations of Malaysia and interpreted that larger leaf size implies higher leaf weight and possibility of higher quantity of leaf sweetener content, but in the present study, after conducting morphological and biochemical analysis we found that the leaf size, leaf length/ width ratio and number of leaves in plant did not show any correlation with the level of stevioside and rebaudioside-A accumulation in the leaves (Tables 2 and 3).

The RAPD markers not only distinguished the difference and similarities among species but also could identify the intra specific diversities, whereas, morphological characteristics could not discern the differences within the species. RAPD markers have been used mainly for the testing the genetic similarity or differences between *in vitro* regenerated and mother population of *S. rebaudiana* (Hassanen and Khalil, 2013), variation between diploid and polyploidy cytotypes (Vanessa et al., 2004; Yadav et al., 2013) and somaclonal variation within regenerated *S. rebaudiana* plants (Moktaduzzaman and Mahbubur Rahman, 2009).

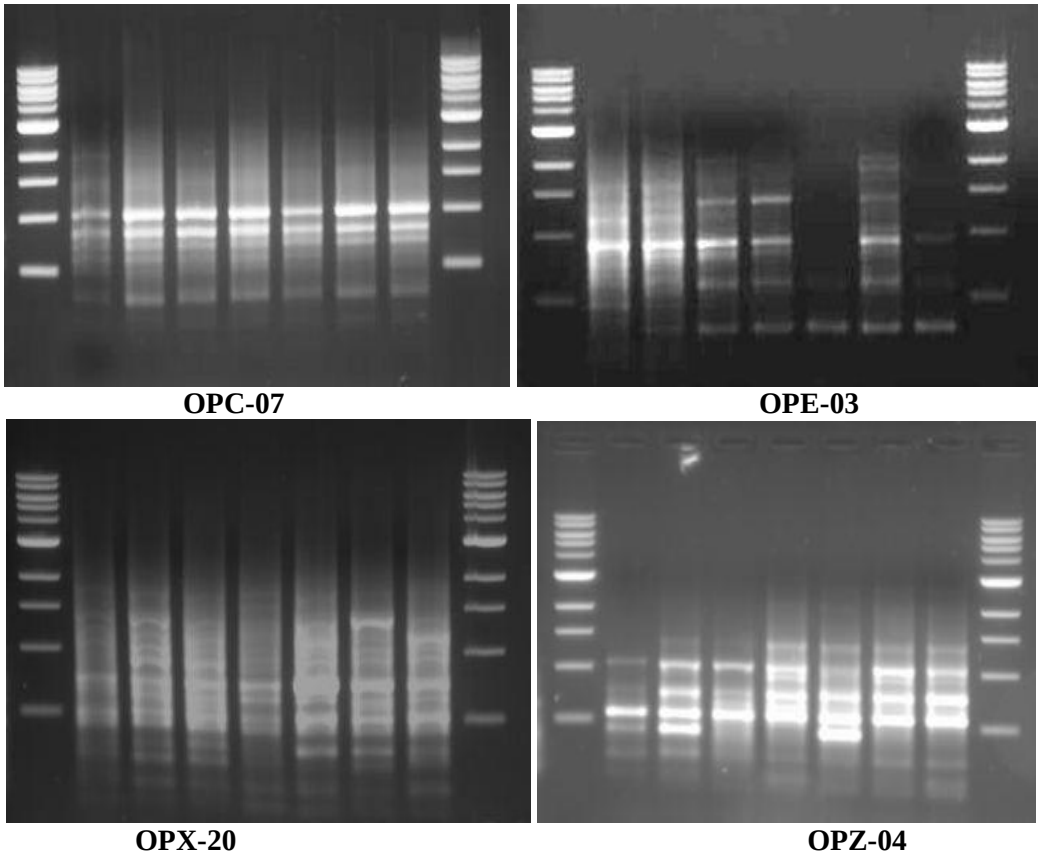

Fig. 3: RAPD polymorphism amongst 7 *Stevia rebaudiana* genotype detected with random RAPD primers: OPC-07, OPE-03, OPX-20 and OPZ-04.

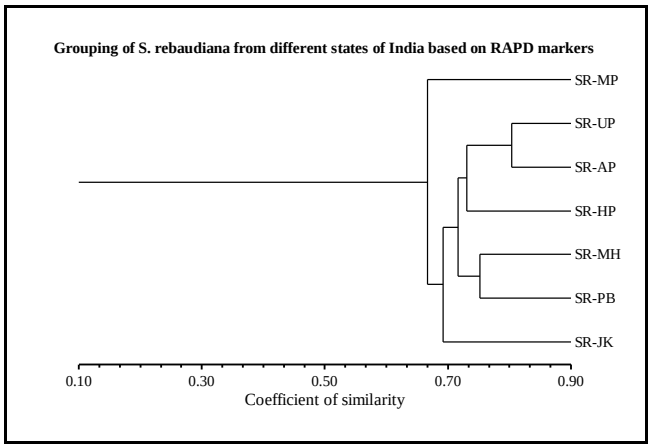

Fig. 4: RAPD markers based dendrogram of *Stevia rebaudiana* ecotypes.

Variations in genetic constitution within the species are usually related with geographic range, mode of reproduction, mating system, seed dispersal and fecundity (Gupta et al., 2008). The genetic diversity detected in the present study may be due to some or all these prevalent factors as the genotype of *S. rebaudiana* studied was widely distributed in different eco-geographical regions. This is in conformity with the study of Thiyagarajan and Venkatachalam (2015) which suggested genetic variation in three accessions of *S. rebaudiana* from different agro climatic regions of India. Morphological, biochemical and molecular characterization of the genetic diversity is crucial for the genetic improvement of stevia to utilize the information to develop an ideal plant type (Yadav et al., 2011).
Diverse Stevia germplasm accessions with natural genetic diversity and considerable contents of rebaudioside and stevioside are of prime interest to breeders for the improvement. Yadav et al. (2011) suggested that Paraguan leaves contained the highest concentration (9-13 %) of the sweet steviosides or rebaudiosides, compared to Chinese stevia with (5-6 %) and Indian stevia containing 9.08% of the dry weight of leaves.

In the present study, stevioside and rebaudioside- A levels differed considerably among the different ecotypes of the *S. rebaudiana*. The amount of stevioside varied from 486.5±35 µg/g of tissue dry weight in SR-MP to 986.8±67 µg/g of tissue dry weight in SR-HP ecotypes whereas Rebaudioside A varied from 175.3±31 to 268.3±44µg/gdw. Among the ecotypes, SR-HP contained significantly high levels of stevioside (986.8±67 µg/gdw) followed by SR-MH (704.5±55 µg/gdw), SR-UP (669.9±46 µg/gdw) and SR-AP (648.5±38 µg/gdw). Least content (486.5±35 µg/gdw) was recorded in SR-MP ecotype (Table 3). Such diversity should be useful in the selection and propagation of *Stevia* germplasm.

In conclusion, the *Stevia rebaudiana* ecotypes collected from different geographical locations of India showed clear variation at morphological, biochemical and genetic level. Genetic diversity analysis through RAPD markers showed that SR-UP, SR-MH, SR-PB and SR-AP are a closely related germplasm, which may be because they are cultivated at the closest latitude area which is further confirmed by stevioside accumulation profile. SR-HP had useful genetic traits in studied ecotypes and could be selected for further tissue culture or cultivation programs of stevia.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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