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Assessment of genetic and biochemical diversity of *Stevia rebaudiana* Bertoni by DNA fingerprinting and HPLC analysis

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Abstract

The genomic DNA polymorphism and phytochemical variation of *Stevia rebaudiana* Bertoni was investigated by RAPD-PCR and HPLC analysis. For initial PCR screening, about hundred oligo-nucleotide primers were used to amplify the genomic DNA from three accessions of *S. rebaudiana* (L1 to L3). The concentration of stevioside content was quantified by using HPLC analysis. A total of 9 primers were finally selected to generate RAPD fingerprints that revealed 111 bands, in which 90 bands (81%) were found to be polymorphic bands. Three accessions were clustered into two major groups in the dendrogram and the stevioside content variation was linearly associated with these groups. This result was further confirmed by principle coordinate analysis. There was also high diversity in the concentration of active compounds in the collected samples as revealed by HPLC analysis. Data revealed that stevioside content varied between 15.82 mg/g DW (L1) to 4.68 mg/g DW (L3). The present results suggested that there was a strong correlation between the phytochemical variables and the DNA polymorphism data. The present study concluded that the results of the genetic and phytochemical diversity could be used to select best accessions for planting and conservation of *S. rebaudiana* for pharmaceutical applications.

Key words: *Stevia rebaudiana* Bertoni, polymorphism, phytochemical, stevioside

1. Introduction

Genetic and environmental factors and their interactions affect the pharmaceutically important secondary metabolites in medicinal plants. A variety of environmental factors, such as season, altitude, radiation, and soil nutrition, have been proven to significantly influence the secondary metabolite profile (Hartmann, 2007). Successful management and conservation of natural populations depend on accurate assessment of genetic diversity to address all questions regarding genetic relationships among individuals as well as levels and structure of genetic variation (Abdel-Mawgood *et al*., 2006). DNA markers are used to assess genetic diversity at various levels of taxon-species, inter and intra population and progeny. At the species level, the knowledge of genetic diversity helps understand the features which make it unique and distinct from other species. Diversity studies at this level help in *ex situ* conservation programmes.

Different types of molecular markers have been used to ascertain DNA polymorphism. One of the most efficient molecular methods in terms of ability to produce abundant polymorphic markers within a short time and low cost is the random amplified polymorphic DNA (RAPD) technique. It is a PCR based technology, a simple and cost-effective tool for analysis of plant genome. It is technically least demanding and offer a fast method for providing information from a large number of loci, particularly in species where no study has previously been undertaken. RAPD is being widely used in various areas of plant research and it 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). More recently, RAPD has been used for estimation of genetic diversity in various plant species such as, *Dendrobium* species (Zha *et al*., 2009), *Zygophyllum* populations (Hammad and Qari, 2010) and *Withania somnifera* (Dharmar and Britto, 2011) and *Cardiospermum halicacabum* (Sheeba *et al*., 2014), collected from different geographical regions.

Stevia rebaudiana Bertoni is a perennial herb, belonging to the family, Asteraceae. *Stevia* is cultivated in several countries in the world, *viz*. Japan, Brazil, Southeast Asia, India, China and Canada. *S. rebaudiana* has attracted economic and scientific interests due to the sweetness and the therapeutic properties of its leaf. Two steviol glycosides present in plant tissue, stevioside and rebaudioside A, are largely responsible for the sweet flavor of stevia leaves. Steviol glycosides are closely related in structure and have both sweet and bitter flavor profiles. Both compounds are approximately 300 times sweeter than sucrose. Stevioside has become well known not only for its intense sweetness but also for offering several therapeutic benefits, as it has antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, antidiarrheal, diuretic and immunomodulator activities (Geuns, 2003; Chan *et al*., 2000; Jeppesen *et al*., 2002). Owing to the growing incidence of obesity and diabetes, stevioside has caught great attention since it has the potential to serve as non-caloric sweeteners. Recently, stevioside has attracted economic interest as a sucrose substitute and the commercial cultivation of *S. rebaudiana* has successfully expanded to several countries. Diterpene glycosides used in variety of foods and products such as pickled vegetables, dried sea food, beverages, candies, chewing gum, yogurt, ice cream, tooth paste, *etc*.

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Several methods are known for determining the quantitative content of glycosides in plant material (*e.g*., gas chromatography or infrared spectroscopy). However, the simplest and most reliable method is HPLC, which has been used to determine the composition of *S. rebaudiana*, growing in various geographical areas (Kovylyaeva *et al*., 2007). Quantitative and qualitative status of active constituents along with genetic diversity in a medicinal plant would help to devise conservation strategies and selection of right sample for maximum yield. The variation in morphological and pharmaceutical characteristics has been attributed to environmental and/or genetic factors. The analysis of population variation based on genetic and chemical markers is necessary for efficient commercial cultivation practices and industrial utilization of plant resources. Also there are reports on correlation between genetic diversity and variation in chemical constituents (Deborah *et al*., 2005; Kuroda *et al*., 2007; Han *et al*., 2008; Tori *et al*., 2008).

The first genetic linkage map for *Stevia* has been constructed based on RAPD markers by Yao *et al*. (1999). Influence of genetic variation on morphological diversity in ten accessions of *S. rebaudiana* in Malaysia was studied by Osman and Abdullateef (2011). Recently, the genetic and metabolic variability in *S. rebaudiana* among accessions of different geographical regions of India, using random amplified polymorphic DNA (RAPD) markers and high-performance thin layer chromatography (HPTLC) analysis was reported by Chester *et al*. (2013). The combination of two methods will be highly useful for understanding the level of secondary metabolite biosynthesis and genetic variability exist within a particular medicinal plant species accessions. Therefore, it is an urgent need to investigate the stevioside contents variation among the different populations to relate the genetic diversity of this important antidiabetic medicinal plant. Genetic diversity of various medicinal plants was positively correlated with the level of bioactive molecules in the recent past (Yuan *et al*., 2007; Ting Han *et al*., 2008; Ali Azizi *et al*., 2012). Hence, it is hypothesized that an increased level of genetic diversity in medicinal plants may have influence on level of bioactive component biosynthesis. The major goal of this study was to correlate the genetic variability with the level of phytochemical content among *Stevia* accessions for commercial applications.

2. Materials and Methods

2.1 Plant materials

A total of three accessions (Table 1) of *Stevia rebaudiana* Bertoni were collected from various locations in Tamil Nadu, India. Young leaves were used for DNA extraction, while excess leaf materials were stored in -80°C for future use. The accessions were chosen to represent a wide geographic range based on the distribution of *S. rebaudiana* in Tamil Nadu, India.

Table 1: Collection locations with altitude, latitude and longitude

Accessions ID	Area of the study / District in Tamil Nadu	Altitude	Latitude	Longitude
L1	Horticulture Research Station(HRS), TNAU, Kodaikanal, Dindigul	2300 m	10°20'N	77°50'E
L2	Horticulture Research Station (HRS), TNAU, Yercaud, Salem Dt. TN	1515 m	11°77'N	78°20'E
L3	Centre for siddha medicinal garden, Mettur, Salem Dt. TN	49 m	11°47'N	77°48'E

2.2 DNA extraction

Total genomic DNA was extracted from leaves, using a modified CTAB method, based on the protocol of Doyle and Doyle (1990). Quality of total DNA was verified by (0.8%) agarose gel electrophoresis, visualized under UV light and image was recorded using gel documentation system (Alpha image, USA).

2.3 DNA concentrations

For each sample, a series of assays were carried out to estimate the optimum DNA concentrations (15-25 ng/µl) for PCR amplifications. This was accomplished by comparing the dilutions to a known DNA standard after separation on 0.8% (w/v) agarose gel and stained with ethidium bromide. The DNA was diluted with sterile TE buffer to 10-15 ng/µL and used for PCR amplification.

2.4 PCR amplifications

RAPD-PCR was carried out according to Williams *et al*. (1990) and the random oligonucleotide primers were obtained from Operon Inc., CA, USA. The reaction was carried out in a volume of 20 µl consisted of 2 µl, 10 × PCR buffer (10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 2 µl, 1.5 mM dNTPs (dATP, dGTP, dCTP and dTTP), 250 nM random decamer primers (1.0 µl), 0.5 units of Taq DNA polymerase, 2 µl of genomic DNA (15 ng) and finally added 13 µl of sterile water. Amplifications were performed in a PCR thermal cycler (Cyber lab, USA) under the PCR amplification profile with initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 1 min/ 37°C for 1.30 seconds/ 72°C for 2 min with a final extension step 72°C for 7 min. After PCR cycles, loading dye was added to the amplified products. The RAPD products were separated on 1.5% (w/v) agarose gel electrophoresis in 1.5% (w/v) containing 0.5 µg/ml ethidium bromide in 1× TAE buffer. Electrophoresis was performed at 60V for about 2 h. until the bromophenol blue dye front had migrated to the bottom of the gel. The molecular standard used was the lambda DNA double digested by *Eco*RI/*Hind* III. The gels were visualized under UV light and photographed with Alpha Inotech gel documentation system (USA). Among primers, those which exhibited clear banding pattern after PCR amplification were selected for further RAPD analysis (Venkatachalam *et al*., 2008).

2.5 Fingerprinting data scoring and analysis

RAPD banding patterns were analyzed by biostatic type scoring. The occurrence of a specific band of amplified DNA was scored as (+) and absence as (–) for all prominent bands within a fingerprint profile. Therefore, a sequence of 'l' and '0' was generated for each primer / species to form a data matrix. DNA fragment sizes were estimated from the agarose gel by comparison with PCR molecular weight marker. A dendrogram was generated by cluster analysis using unweighted paired group method with arithmetic average (UPGMA) based on Jaccard's similarity co-efficient. The fit of dendrograms obtained were checked by bootstrapping.

2.6 Phylogenetic tree analysis

To evaluate the genetic relationship between different locations of *Gymnema* plant species, DNA bands from seven accessions produced by different random primers were scored and used for construction of phylogenic tree. The lambda DNA double digested by *EcoR I/Hind* III was used to determine the size of each amplified DNA fragment. RAPD fragments were assigned a DNA length and

recorded as a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *S. rebaudiana* plant species was generated, using Hierarchical clustering of DARwin 5.0 software based on UPGMA method.

2.7 Extraction of sweet diterpene glycosides from *S. rebaudiana*

Shade dried and ground leaves of *S. rebaudiana* (5 g) were placed in 250 mL conical flasks and extracted by shaking for 30 min with 100 mL of EtOH 70% (w/v) in a 70°C water bath. After cooling, a 10 mL aliquot was filtered using Whatman filter paper (No.1), and then 20 µL was analyzed by HPLC.

2.8 HPLC analysis

HPLC analysis was performed on a Shimadzu (Tokyo, Japan) model LC10A instrument equipped with Shimadzu, A Ch1 photodiode array detector (PDA). The mobile phase was acetonitrile: water (80:20) at a flow rate of 0.6 ml/min. The detection wavelength was 210 nm which was close to the absorption maxima for compound. The injection volume for sample was 20 µl and column temperature was 26°C.

3. Results and Discussion

Genomic DNA polymorphism of *S. rebuadiana* Bertoni accessions was accessed by RAPD-PCR analysis. In a preliminary screening, hundred oligonucleotide primers were used to amplify the genomic DNA isolated from three accessions of *S. rebaudiana* plant species (L1 to L3). Out of hundred primers screened, only nine RAPD primers (OPA10, OPA13, OPA16, OPB01, OPB07, OPC09, OPC15, OPC16 and OPC19), produced clear and reproducible DNA fragments among the three accessions of *S. rebaudiana* plant species. The selected primers produced 111 bands and 90 bands were showed polymorphisms. The highest number of RAPD bands was detected in OPC16 primer (16 bands) and followed by OPA10 (13 bands) while the lowest number of DNA bands was observed with OPA13 (4 bands) (Figure 1). The DNA finger printing profile for three accessions of *S. rebaudiana* is showed in Table 2. The percent of polymorphic bands obtained for each primer was 81%OPA10, 44%(OPA13), 100%(OPA16), 77%(OPB01), 83%(OPB07), 85%(OPC09), 91%(OPC15), 80%(OPC16) and 83%(OPC19). It is interesting to note that 100% polymorphism was noticed with primer OPA16 compared to other primer.

In OPA10 primer produced RAPD profiles have a specific 1100 bp DNA fragment in L1 accessions which was absent in L2 and L3 accessions. In case of OPC16 primer, L1 accessions produced a specific DNA fragment with approximate size 1662 bp, but it was not amplified in L2 and L3 accessions (Figure 1).

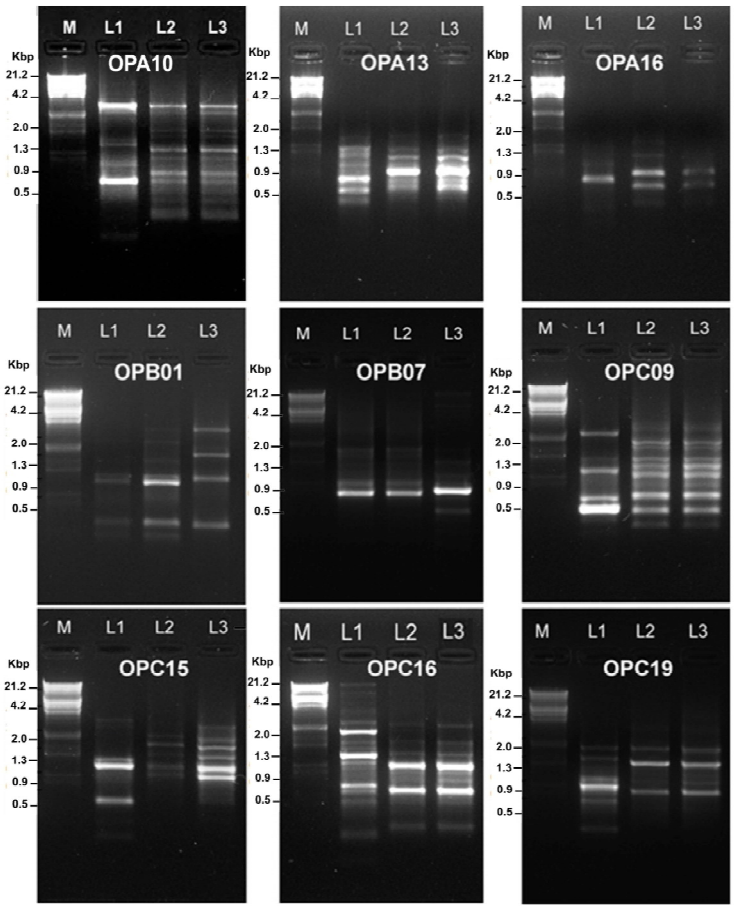

Figure 1: RAPD pattern generated using various selected oligonuclotide primers. (Accession ID: L1-Kodaikanal; L2-Yercaud; L3-Mettur)

Table 2: RAPD primers, products, their sequences generated by random amplified polymorphic DNA (RAPD) primers in *Stevia rebaudiana* Bertoni collected from different locations of Tamil Nadu

Primers	Nucleotide sequence 5' to 3'	Total No. of bands	No. of polymorphic bands	Polymorphism (%)	Banding range (base pair)
OPA10	GTGATCGCAG	16	13	81	2620- 250
OPA13	CAGCACCCAC	9	4	44	1171-300
OPA16	AGCCAGCGAA	7	7	100	1096-250
OPB01	GTTTCGCTCC	9	7	77	2911-564
OPB07	GGTGACGCAG	12	10	83	1801-300
OPC09	CTCACCGTCC	14	12	85	2227-300
OPC15	GACGGATCAG	12	11	91	2728-250
OPC16	CACACTCCAG	20	16	80	2163-250
OPC19	GTTGCCAGCC	12	10	83	2778-250
	Total	111	90	80.44	

3.1 Phylogenetic analysis

To evaluate the genetic relationship between different locations of *S. rebaudiana*, DNA bands from three accessions produced by random primers were scored and used for construction of phylogenic tree. RAPD fragments were assigned a DNA length and recorded as a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *S. rebaudiana* was constructed using Hierarchical clustering of DARwin 5.0 software based on UPGMA method (Figure 2).

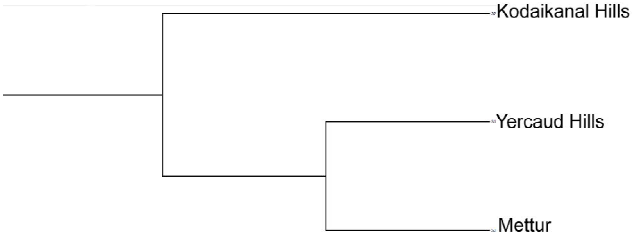

Figure 2: Unweighted pair group method with arithmetic average (UPGMA) tree showing the genetic relationships between three accessions of *S. rebaudiana* Bertoni as determined by RAPD markers

A dendrogram of genetic similarities among the three populations was constructed, using the genetic distance coefficients shown in Table 3. Based on the clustering analyses, we classified the populations into two major clusters: Group I (Accessions L1) and Group II (Accessions L2 and L3). The present results showed that the variation exist among the different accessions of *S. rebuaidana* which can be utilized for the production of quality herbal formulations.

Table 3: Jaccard coefficient similarity index of three accessions of *Stevia* species based on RAPD markers

Accession ID	L1	L2	L3
L1	0.0000		
L2	0.6923	0.0000	
L3	0.7904	0.3333	0.0000

(Accession ID: L1-Kodaikanal; L2-Yercaud; L3-Mettur)

DNA molecular markers are useful genetic markers and, hence, may have diverse applications (Liang *et al.*, 2001). Their application in the study of medicinal plant species would enhance the accuracy of selecting better accessions and accelerate both their commercial cultivation and industrial applications (Li and Tu, 2003).

The present study revealed that genetic relationships among three *Stevia* populations, using DNA polymorphism data, classified the populations into two major clusters, and found correlations among genetic makeup and place of origin of the populations. These findings may be highly beneficial for protecting genetic resources and promoting sustainable use of *S. rebaudiana* in pharmaceutical industry.

3.2 HPLC analysis

The concentration of stevioside content of the *S. rebaudiana* was quantified by HPLC analysis and given as mg/g of dry weight (DW) tissue (Table 4). Chromatograms of the stevioside standard and representative accessions are displayed in Figure 3. The relative concentrations of stevioside within the accessions and the stevioside compositions are presented for the leaves within each accession. The levels of stevioside from individual accessions of the *S. rebaudiabana* varied substantially. The stevioside concentrations of individual populations were 15.82 mg/g DW for L1; 9.726 mg/g DW for L2; 4.689 mg/g DW for L3. Maximum variation was also apparent of stevioside composition ranged from 15.82 mg/g DW (L1) to 4.689 mg/g DW for L3. The present study strongly confirmed that, the results of the individual analysis illustrate the significant stevioside variation to be found within the *S. rebaudiana* accessions and its implication of pharmacological research and development.

Table 4: Contents of stevioside in different accessions of *S. rebaudiana* Bertoni

Accessions ID	Area of the study / District in Tamil Nadu	Stevioside content (mg/g DW) (Mean ± SE)
L1	Horticulture Research Station (HRS), TNAU, Kodaikanal, Dindigul	15.82±1.08[a]
L2	Horticulture Research Station (HRS), TNAU, Yercaud, Salem Dt, TN,	9.726±0.98[b]
L3	Centre for Siddha medicinal garden, Mettur, Salem Dt.	4.689±1.37[c]

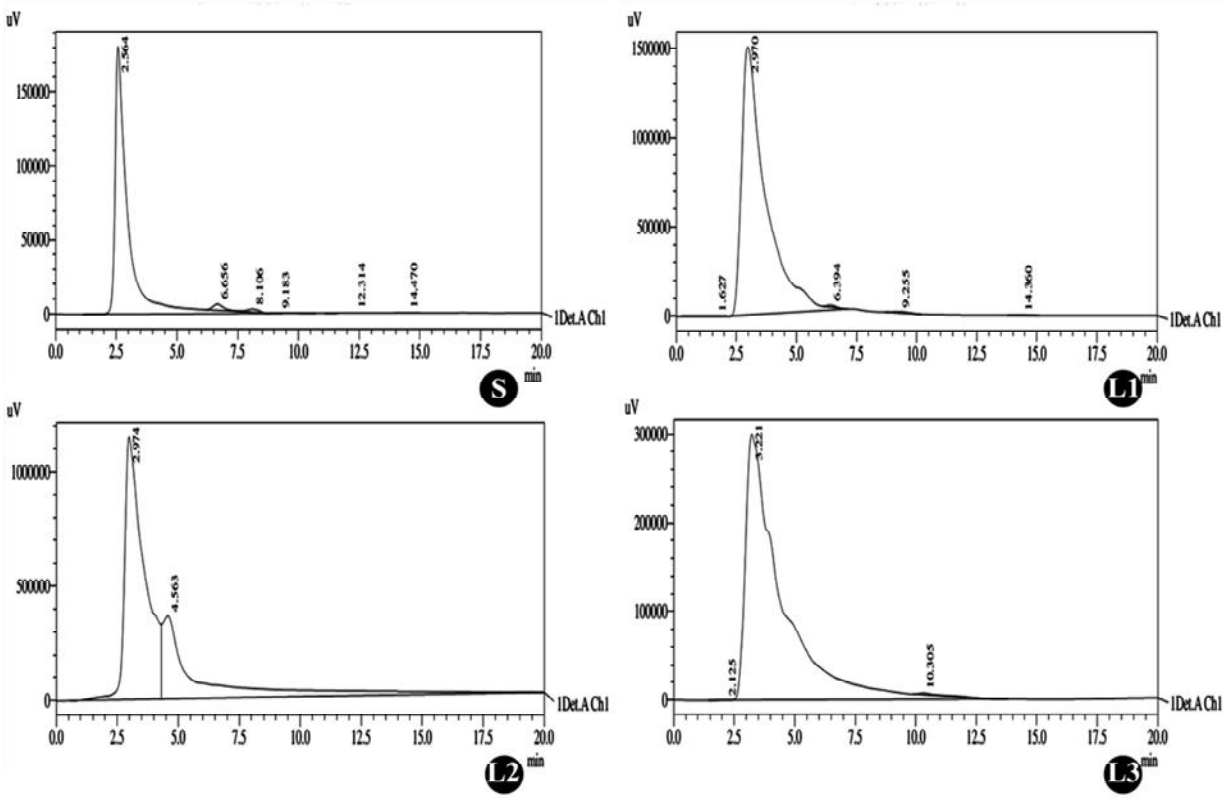

Figure 3: HPLC stevioside chromatograms of *S. rebaudiana* Bertoni chemotypes. Chromatogram of stevioside standards (S), Kodaikanal (L1), Yercaud (L2) and Mettur (L3) accessions

The PCoA analysis revealed more information about the stevioside content at various altitudes. Firstly, the content variation was proved to be related to altitude. Samples at a high altitude always contained more stevioside content. For example, the average content of steviosides accessions L1 was about 3 times higher than in accessions L3. Secondly, the analysis revealed the best accessions for the production of higher quantity of stevioside. The best region to accumulate the stevioside content was 2510 m. Finally, the *Stevia* samples collected at low altitude had remarkably decreased level of stevioside contents. The present results showed that high altitude can accumulate stevioside contents in plants. Based on the above mentioned conditions, the content variation of steviosides was further speculated to relate with altitude increase, which lead to temperature drop, resulting in the difference of biosynthesis and accumulation of steviosides. According to correlation analysis, altitude was found to be main factors that influence the stevioside content production in *S. rebaudiana.* Similar observation was also reported recently in *Rheum rhabarbarum* (Zhe Wang *et al*., 2013).

3.3 Genetic distances and chemotype

In the present study, nine RAPD primers that yielded 90 highly reproducible polymorphic markers known to effectively discriminate among *S. rebaudiana* accessions were used to calculate unweighted pair group method with arithmetic mean (UPGMA) genetic distances. A dendogram of neighbor-joining cluster analysis of the genetic distances shows a close correspondence between chemotype and RAPD profiles. The ratios of the common DNA markers and genetic distance coefficients among the populations were calculated using Jaccard co-efficient. The genetic distance coefficients among the populations were distributed between 0.3333 and 0.7904 (Table 3).

For principal coordinates analysis (PCoA), dissimilarity matrix values, which were calculated using a binary data matrix with Jaccard co-efficient, were used to ordinate accessions from 3 different *Stevia* populations on a scattered plots. The genetic distance values were used for hirarical cluster analysis using UPGMA agglomeration method using Darwin 5.0.

According to PCoA results, the codes of the accessions from each population were plotted on the four components corresponding to different regions from where the 3 different *Stevia* populations were collected from Tamil Nadu. The PCoA displayed the separation of populations (L1), (L2) and (L3) according to genetic distances and based on dissimilarities matrix values on a scattered plot graph (Figure 4).

Correlation between chemotype and RAPD profiles was clearly displayed in four dimensions PCoA scattered plot. Kodaikanal accessions (L1) clearly separates from other two accessions (L2 and L3) of *S. rebaudiana*. The analysis of RAPD-based genetic distances and chemotype, which provides the molecular evidence that stevioside variation in *S. rebaudiana* had a strong genetic basis. It is reported that there was a close association between genetic and chemotypic profiles of *Panax quinquefolius* (Schlag and McIntosh, 2013).

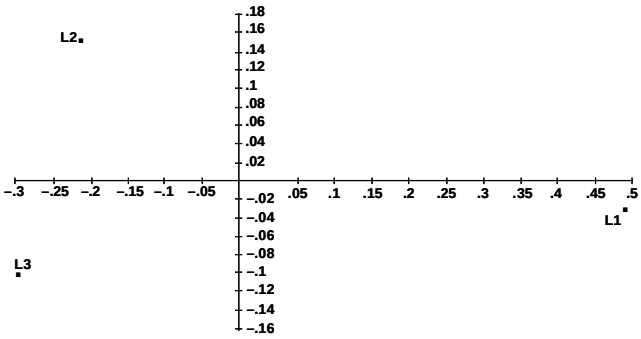

Figure 4: Principal coordinates analysis of RAPD markers from three accessions of *S. rebaudiana* (Accession ID:1-Kodaika-nal; 2-Yercaud; 3-Mettur)

4. Conclusion

The present study concludes that, genetic diversity linearly correlated to the concentration of the bioactive constituents (stevioside) present in the plant samples. Among the *S. rebaudiana* accessions tested, L1 Kodaikanal accessions may be more suitable for cultivation and production of high yield of stevioside content followed by L2 Yercaud accessions. The correlation between RAPD markers and chemotype indicates the potential to use RAPD analysis as a reliable method for identification and authentication of high yield accessions of pharmaceutically important medicinal plants including *S. rebaudiana* for future industrial applications.

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Conflict of interest

We declare that we have no conflict of interest.

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