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Assessment of genetic diversity of *Stevia rebaudiana* Bertoni by DNA fingerprinting

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Abstract
The genomic DNA polymorphism variation of *Stevia rebaudiana* Bertoni was investigated by RAPD-PCR analysis. For initial PCR screening, about hundred Oligo-nucleotide primers were used to amplify the genomic DNA from three accessions of *Stevia rebaudiana* (S1 to S5). A total of a primer were finally selected to generated RAPD fingerprints that revealed III bands, which 88 bands (80%) were found to be polymorphic bands. Five accessions were clustered in to two major groups in the dendrogram. The present results suggested that there was strong correlation between the variables in DNA polymorphism data. The present study concluded that the results of the genetic diversity could be used to select best accessions for planting and conservation of the *Stevia rebaudiana* pharmaceutical applications.

Keywords: *Stevia rebaudiana*, RAPD-PCR, polymorphism

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 nutrients, have been proven to significantly influence the secondary metabolite profile (Heartmann, 2007). Successful management and conservation of natural population 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) [1]. DNA markers are used to assess genetic diversity at various levels of taxon –species, inter and intro population and progeny. At the species level, the know 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 programs. Different types of molecular markers have been used to ascertain DNA polymorphisms. One of the most efficient molecular methods in terms of ability to produce abundant 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 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 gene (Kuddus *et al.*, 2002) [6]. The first linkage map of *steiva* has been constructed based on RAPD makers by Yao *et al.*, (1999) [12]. Influence of genetic variation on morphological diversity in ten accessions of s rebaudiana in malaysia was studied by Osman and abdullateef (2011) [9]. Recently the genetic and metabolic variability in s rebaudiana among accessions of different geographical regions of india, using random amplified polymorphic DNA (RAPD) markers has been reported by chester *et al.*, (2013) [3]. 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 a urgent need to investigate the 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 (Ting Han 2008, Ali Azizi *et al.*, 2012) [10, 2]. 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.

Material and Methods
Plant materials
A total of five 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 *Stevia rebaudiana* in Tamil Nadu, India.

Table 1: Collection locations with altitude, latitude and longitude

Accessions ID	Areas of the study District in Tamil Nadu	Altitude	Latitude	Longitude
S1	Horticulture Research Station (HRS), TNAU Kodikanal, Dindigul	2300 m	10˚20' N	77˚50'E
S2	Centre for siddha medicinal garden, Meetur, Salem, Dt, TN	49 m	11˚47' N	77˚48'E
S3	Horticulture Research Station (HRS), TNAU Yercurd, Salem, Dt, TN	1515 m	11˚77' N	78˚20' E
S4	Government Botanical Garden, Ooty, TN	2500m	11.4189'N	76.7114'E
S5	Kollimalai hill, Namakkal, Dt, TN	1400m	11.2485'N	78.3387'E

DNA extraction

Total genomic DNA was extracted from leaves, using a modified CTAB method based on the protocol of Doyle and Doyle (1990) [4]. 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).

DNA concentration

For each sample, a series of assay were carried out to estimate the optimum DNA concentration (12-20ng/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 diluted with sterile TE buffer 10-15ng/µL and used for PCR amplification.

PCR amplifications

RAPD-PCR was carried out according to willams *et al*., (1990) and the random oligonucleotide primers were obtained from Operon Ino., CA, USA. The reaction was carried out in a volume of 20µl consisted of 2µl, 10 x PCR buffer 10 Mm Tris HCl (Ph 8.3), 50Mm KCl, 1.5Mm $MgCl_2$), 2 µl, 1,5 mM dNTPs (dATP, dGTP, dCTP, and dTTP) 250 nM random decamer primer (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 under the PCR amplification profile with initial denaturation at 94 ˚C for 4min, followed by 40 cycles at 94 ˚C for 1min/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 0.5µg/ml ethidium bromide in 1x TAB buffer. Electrophoresis was performed at 60V for about 2h, 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 EcoRI/Hind III. The gel were visualized under UV light and photographed. Among primers, those which exhibited clear banding pattern after PCR amplification were selected for further RAPD analysis (Venkatachalam *et al*., 2008) [11].

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 with in a finger print profile. Therefore, a sequence of 'p' and 'o' was generated for each primer species to form a data matrix. DNA fragments 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 obtaind were checked by bootstrapping.

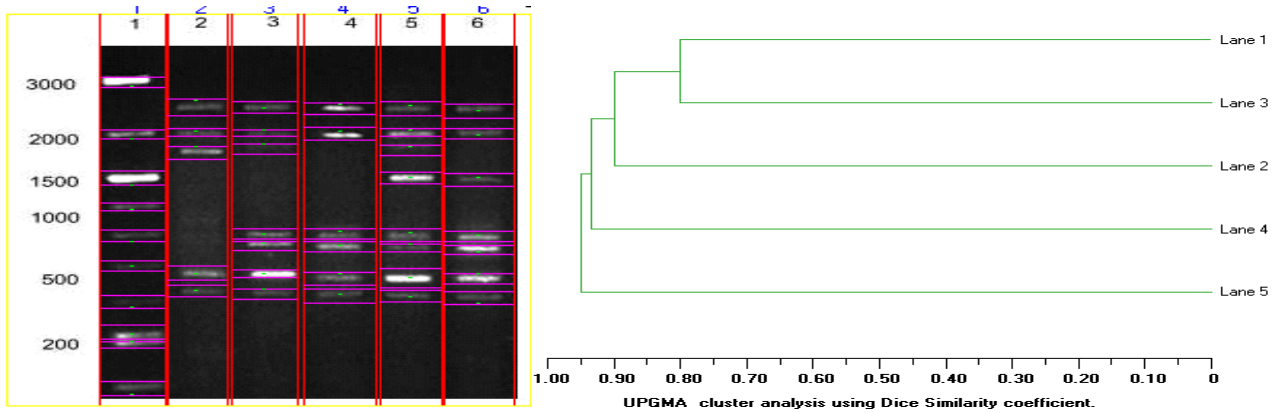
Phylogenetic tree analysis

To evaluate the genetic relationship between different location of *Gymnema* plant species, DNA bands from seven accessions produced by different random primers were scored and used for construction of phylogenetic 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.

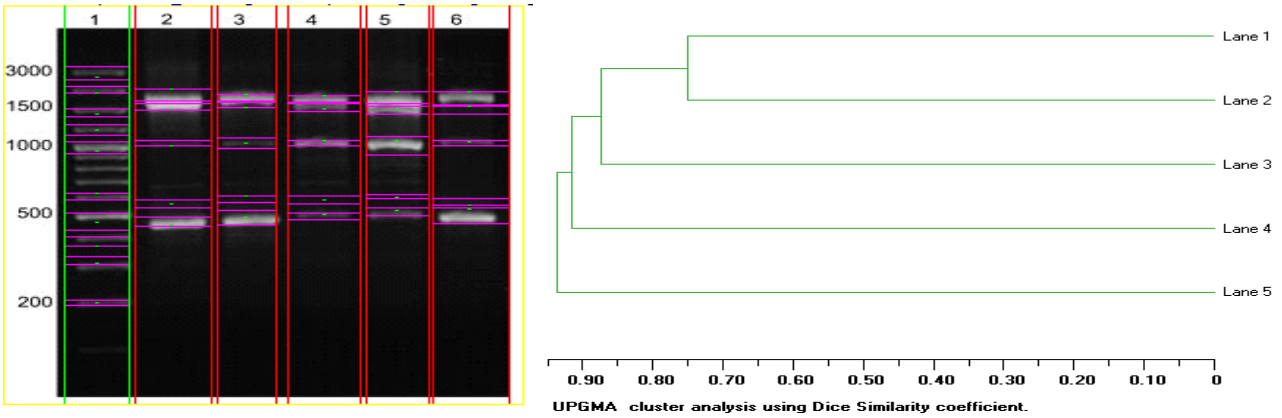
Results and Discussion

Genomic DNA polymorphism of S. *rebaudiana* Bertoni accessions was accessed by RAPD-PCR analysis. In a preliminary screening hundred oligonuclotide primers were used to ampilify the genomic DNA isolated from three accessions of *S. rebaudiana* plant species (L1-L5). 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 five accessions of *S. rebaudiana* plant species. The selected primers produced 108 bands and 88 bands were showed polymorphiams. The highest number of RAPD bands was detected in OPC 16 primers (16 bands) and followed by OPA 10 (13 bands) while the lowest number of DNA bands was observed with OPA 13 (4 bands) (Figure -1). The DNA finger printing profile for five accessions of 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.

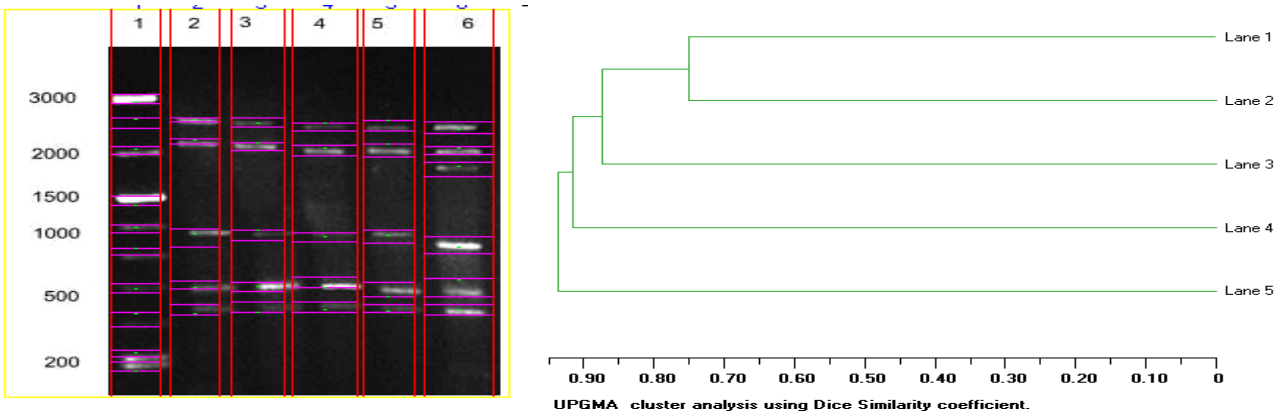
In OPA10 primer produced RAPD profiles have a specific 1100 bp DNA fragment in S1 accessions which was absent in S2, S3, S4 and S5 accessions. In case of OPC16 primer, S1 acccessions produced a specific DNA fragment with approximate size 1772bp, but it was not amplified in S2, S3, S4 and S5 accessions (figure -1).



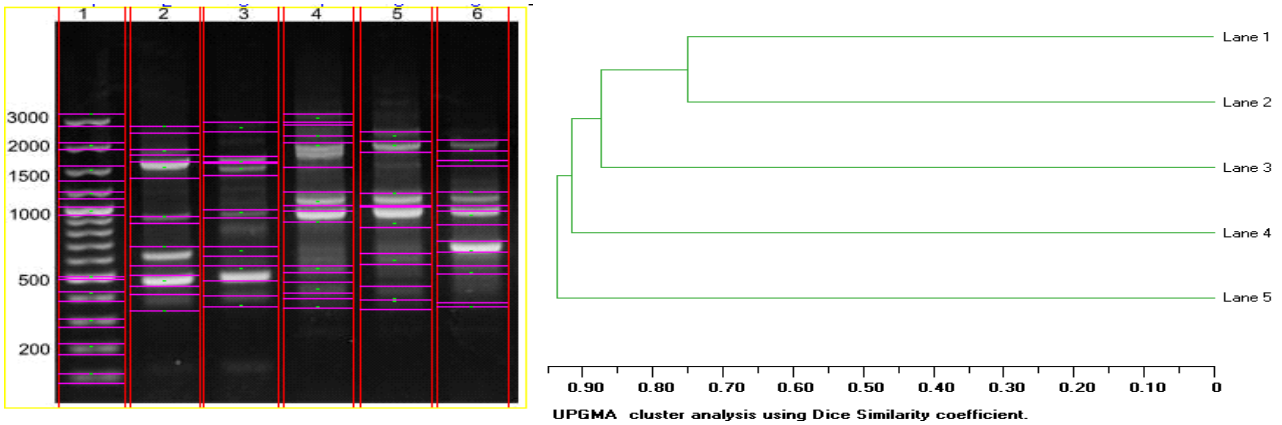
Sample 1



Sample 2



Sample 3



Sample 4

Fig 1: RAPD pattern generated using various selected oligonucleotide primers.

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

Phylogenetic analysis

To evaluate the genetic relationship between different locations of *S. rebaudiana*, DNA bands from five 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).

A dendrogram of genetic similarities among the five populations was constructed, using the genetic distance coefficients. Based on the clustering analysis, we classified the populations into two major clusters: Group I (Accessions S1) and Group II (Accessions S2, S3, S4 and S5). The present results showed that the variation exist among the different accessions of *S. rebaudiana* which can be utilized for the production of quality herbal formulations. DNA molecular markers are useful genetic marker and hence may have diverse applications (Liang et al., 2001) [8]. Their applications 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 relationship among five 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.

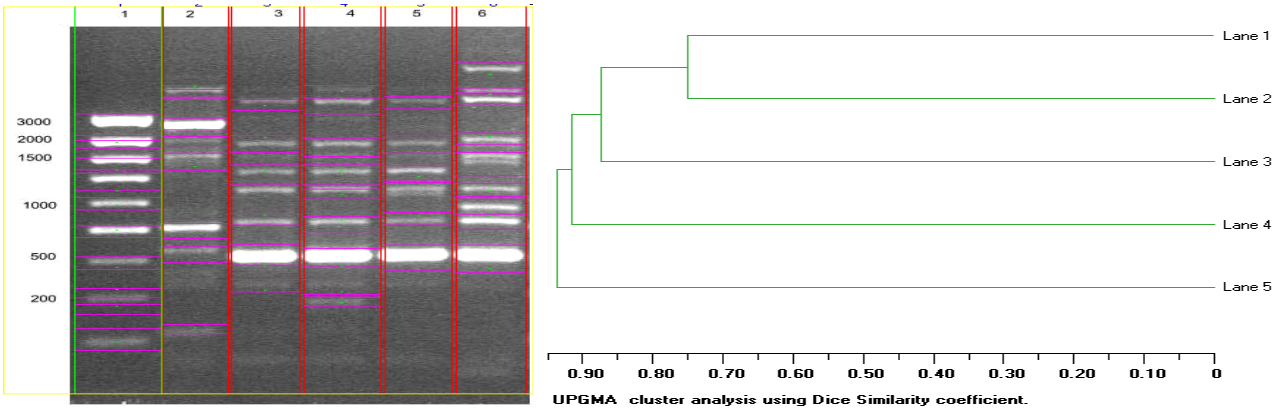


Fig 2: Unweighted pair group method with arithmetic average (UPGMA) five showing the genetic relationship between five accessions of *S. rebaudiana* Bertoni as determined by RAPD markers

Conclusion

The present study concludes that, genetic diversity linearly correlated to the concentration of the bioactive constituents of (stevioside) may be present in the plant samples. Among the *S. rebaudiana* accessions tested, S1Kodaikanal accessions may be more suitable for cultivation and production of high yield of bioactive compound content followed by S3 Yercaud accessions. The correlation between RAPD markers 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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