BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2018. Vol. **15**(4), p. 949-955

Phylogenetic Assessment of Garcinia Species Using RAPD Markers

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http://dx.doi.org/10.13005/bbra/2706

(Received: 09 October 2018; accepted: 12 December 2018)

The plants in the Garcinia species are economically important.Phylogenetic investigation is needed for these tree species to boost breeding and conservation programmes. Six *Garcinia* species were investigated for their phylogenetic relationship using Random Amplified Polymorphic DNA(RAPD) markers.A standardised procedure was developed for isolation of DNA from the leaf samples of *G. cambogia, G. indica, G. xanthochymus, G. morella, G. mangostana* and *G. livingstonei.* Phylogenetic investigation is needed for these tree species to boost breeding and conservation programmes. A standardised procedure was developed for isolation of DNA from the leaf samples of *G. cambogia, G. indica, G. xanthochymus, G. morella, G. mangostana* and *G. livingstonei.* The DNA samples were subjected to PCR using 8 random primers. 269 polymorphic bands were obtained and scored to develop the values for the genetic distance. The dendrogram was developed using the software dendro UPGMA and the Cophenetic correlation coefficient of 0.801 is obtained. *G. cambogia* and *G. livingstonei* are closely placed with a score of 24% followed by *G. morella.* It had a 30% index score to *G. cambogia* and *G. livingstonei* but is followed by just 31% score with *G. indica. G.mangostana* is connected at 33.5% dissimilarity to the above groups showing it is an introduced variety. *G. xanthochymus* is the last link with 37% score in the matrix. The data represented is the first of the type for the species.This will help in further DNA related work in these species. The genetic relatedness among these species is reported and this can be utilised in marker analysis for other Garcinia species.

Keywords: Garcinia; RAPD; phylogenetic analysis.

Garcinia is a group of plant species comprising of 250 species belonging to family *Clusiaceae*. The species are well distributed in the Asian continent. The species of Garcinia in India are distributed in the Western Ghats and the North East regions. Of the 30-35 species reported in India from the Western Ghats, few are endemic in nature.[1] The endemic varieties are mostly trees and are very important plant species of the forests.[2] Cultivation of a few species is seen in Kerala and Karnataka states. These trees contain fruits that are utilised for the economical and pharmacological properties. *Garcinia cambogia* (Gaertn.)Desr., *Garcinia indica* (Thouars) Choisy, *Garcinia xanthochymus* Hook. f. ex J.Anders, *Garcinia morella* (Gaertn.)Desr, *Garcinia mangostana*L. and *Garcinia livingstonei* T. Andersonare the common varieties that are seen distributed in these regions. *Garciniamangostana*

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is an introduced variety from the Indonesian region.[3] *Garciniacambogia (G. gummi-gutta* L. Roxb.) are medium sized trees and the fruits are used to extract Hydroxycitric acid(HCA). HCA is used in anti-obese drug formulations.[4] The fruits from *Garcinia indica,* also a medium sized tree is used in culinary, cosmetics and pharmaceutical formulations.[5] *Garciniaxanthochymus* twigs contains important phenolic compounds used in anti-cancer treatment.[6] *Garciniamorella* is utilised for a phytochemical: morellin.[7] *Garcinia mangostana*was introduced in India for its importance as an edible fruit.[8] *Garcinialiving stonei* fruits contain bioflavonoids that are used in treatment for colon cancer in humans.[9]

Randomly Amplified Polymorphic DNA (RAPD) markers are PCR-based markers that amplify random regions on DNA segments. The primers used are short, the techniques are fast, and it uses very less DNA as template. There is no prior knowledge needed of the sequences from these species.[10] PCR involves a procedure to amplify specific segments of DNA. A combination of RAPD primers in PCR help us to get amplified DNA segments from the plant template DNA provided for the investigation. The plants in the Garcinia genus of late has been investigated in isolated populations for their phylogenetic relationshipsISSR analysis in *G.mangostana* in Sumatra region has been established.[11] The molecular data is used to validate endemicity of varieties and to rule out possible introductions in the past. Phylogenetic analysis among the species can help researchers understand evolutionary pathways of genes, genomes and species by utilizing genetic differences.

There are different varieties of *Garcinia cambogia* being cultivated in different regions.[12] Breeding programs have been initiated in various areas.[13] Selection based on morphological data is tedious and time taking. Several attempts have been made to identify and develop SCAR based markers for sex determination and plant identification.[14]

The objective of the present investigation was to identify genetic relationship among the six species using RAPD markers. Polymorphism data was analysed and genetic relatedness among the six species was recorded. DNA extraction and PCR protocols have been standardised for *Garcinia indica* and this procedure can be adapted to tree species.[15] The standardisation of DNA extraction, PCR protocols, ISSR and RAPD marker analysis in other *Garcinia* species is also investigated.[16] No genetic relatedness is derived out of the investigation. Genetic diversity in eight Garcinia cambogia genotypes was analysed by PCR-RAPD method and genetic relatedness and diversity has been recorded.[17] Genetic diversity relates to understanding the species ability to adapt to the environment.[18] The molecular data is limited and an attempt to develop a relationship study among the species is made. In the present investigation bands obtained from the PCR-RAPD gels were scored and a dendrogram constructed based on the unweighted pair group method with arithmetic mean (UPGMA) clustering method is developed. The molecular data is limited and an attempt to develop a relationship study among the species is made. The result will add on to the molecular data of these tree species. A Cophenetic Correlation can be obtained for these species and the report on its closeness in cluster form RAPD markers can be established. This data will support the phenotypic relatedness among species.

MATERIAL AND METHODS

Collection of leaf samples

The trees of *G. cambogia, G. indica, G. xanthochymus, G. morella, G. mangostana* and *G. livingstonei* used in the study were located and the GPS coordinates of the collection spots are recorded in Table 1. The collection areas recorded are fromsites which are close to each other. The collection herbarium is recorded at the department of Biotechnology, St. Joseph's college, Bangalore. Leaf samples were collected and stored in liquid nitrogen.

500 mg of the leaf sample were homogenised using tissue homogenizer with 15 ml of lysis buffer consisting of 2% Hexadecyltrimethyl ammonium bromide (CTAB), 100mM Tris pH 8, 20m M EDTA, 1.4M NaCl, 2% polyvinylpyrrolidone 40 (PVP) and 0.2% Beta-mercaptoethanol. The extract was transferred to a tube and were incubated at 65°C for 1 hour in a water bath. A set of six tubes with the six species was obtained. The tubes are Centrifuged at 10000 rpm for 10 minutes. Supernatant was

transferred to a fresh tube. 12ml is recovered from the supernatant. Equal volume of Chloroform was added, and the tubes are mixed.The tubes are than centrifuged at 10000 rpm for 15 minutes. The aqueous layer is pipetted out into fresh centrifuge tube without disturbing the interface. Equal volume of Isopropanol and 2 ml of 3M Sodium acetate was added. The contents with the tube are kept at room temperature for 10 minutes. The tube is than centrifuged at 10000 rpm for 15 minutes. The supernatant was discarded. The pellet was washed with 600 µl of 75% ethanol. The pellet air dried and stored in 600µl of 1X Tris- EDTA buffer. The method was used to extract the DNA from all the six species of *Garcinia* leaf samples.

Column purification

Plant genomic purification kit, with the catalogue no 2115700021730 from Genei laboratories, Bangalore India was used to purify the extracted DNA samples.The quality of isolated genomic DNA was checked by Agarose gel electrophoresis. 2µl of DNA was mixed with 1µl of 1X loading dyeand loaded in a slot of 0.8% agarose gel containing 0.015 µg/ml of Ethidium Bromide. The purified samples were subjected to RAPD-PCR reactions.

PCR-RAPD analysis

The PCR reaction was set with the components detailed in table 2. 37µl of this reaction mixture was aliquoted into 48 different labeled PCR vials and to this 2 µl of different template DNA and 1µl random primer were added. The details of the eight RAPD primers used is detailed in table 3. The PCR was set in Eppendorf Mastercycler Gradient unit with reaction conditions detailed in table 4.

Table 1. GPS coordinates of plant specimens.

Plant species	GPS coordinates	
Garcinia cambogia	12°05’26.0"N	76°02’03.1"E
Garcinia indica	12°05’30.8"N	76°01’59.3"E
Garcinia xanthochymus	12°05’33.0"N	76°01’48.5"E
Garcinia morella	11°59’41.5"N	76°04’01.9"E
Garcinia mangostana	$11^0$59’40.2"N	76°04’02.6"E
Garcinia livingstonei	12°05’24.9"N	76°01’56.5"E

Table 2. PCR reaction components

Components	Master mix 1X	Master mix 48X
D.D.H20	17ul	816ul
2X PCR Master MIX	20 µl	960µl
Random Primer	1µl	48µl
Template DNA	2ul	96ul
Total Volume	40 µl	1920µl

Table 3. List of RAPD Primer with sequence details

Sl.no	Primers	Sequence
1.	OPA-02	TGCCGAGCTG
2.	OPB-10	CTGCTGGGAC
3.	OPD-02	GGACCCAACC
4.	OPC-06	GAACGGACTC
5.	OPD-08	GTGTGCCCCA
6.	OPC-07	GTCCCGACGA
7.	OPB-07	GGTGACGCAG
8.	OPB-08	GTCCACACGG

After the reaction the components in the vial were loaded on agarose gels.

Detection by Agarose gel electrophoresis

The RAPD-PCR products were separated based on their molecular weight, by agarose gel electrophoresis. 1.2% agarose gel with 0.015 µg/ml of Ethidium Bromide were used. The DNA were run using electrophoresis buffer and in electrophoresis units and observed under gel documentation units. DNA ladder 100 bp was used as molecular size marker. The gel was documented

Table 4. PCR Cycle conditions

Temperature	Time	No. of cycles
94°C	5 minutes	1
94°C	30 second	40
45°C	1 minute	
72°C	1.30 minute	
72°C	7 minutes	1
4°C	Till loading	

in Gel documentation unit and photographs of gels displaying the bands were taken for data analysis.

Data analysis

Each amplified product from the gel was scored as a unit character and the populations were recorded for the presence (1) or absence (0) of a band. By using the concept of calculating all possible pair-wise genetic distances, the values were obtained from the following formula: D_{ab} = 1"($2n_{ab}$/(n_a+n_b)), Where, n_a and n_b are the numbers of bands amplified in individuals a and b respectively and $2n_{ab}$ is the number of bands shared by those individuals.[19,20,21]The percent disagreement between the populations was calculated based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The resulting dissimilarity index was used to evaluate the relationship among various populations of this species with cluster analysis, using Unweighted pair-group average. All computations were carried out using the phylogenetic software, Dendro UPGMA.[22,23]

RESULT AND DISCUSSION

The DNA was successfully extracted from all the six Garcinia species. The extracted DNA samples were column purified and checked on agarose gel (Figure 1). RAPD amplicons were resolved on agarose gels (Figure 2,3). A total of 271 bands were obtained and scored from 8 primers of which, 269 were polymorphic (Table 5). The results showed 98% polymorphic bands for the primer OPD-02 and 100% for the other primers. The only monomorphic band was of2.4kb and was obtained from the OPD-02 primer having a sequence GGACCCAACC. The scoring data from the bands were analysed for the values for pair wise distance matrix calculation. The generated values are reported in a distance matrix format in Table 6. From the values of the distance matrix, the software DendroUPGMA generated the dendrogram as represented in Figure 4. The dendrogram is also represented in Newickformat,a way of representing dendrograms as a text, using parentheses and commas.[24]All the tree-visualization programs accept this format. The Newickrepresentation for thedendrogram obtained is:(((((Ca:0.243, li:0.243):0.067, mo:0.309):0.009 ,in:0.318):0.017, ma:0.335):0.034,xa:0.370). The Cophenetic correlation coefficient (CP) from the data of RAPD scoring for was found to be 0.80. The CP value for this dendrogram is a measure of how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data points. It is a value between 0 and 1, where 1 represents a perfect match in the dendrogram

Table 5. Percentage polymorphism of primers

Primers	No of bands -A	No of polymorphic bands -B	% polymorphism B/A x 100
OPA-02	21	21	100
OPB-10	29	29	100
OPD-02	50	49	98
OPC-06	31	31	100
OPD-08	28	28	100
OPC-07	49	49	100
OPB-07	26	26	100
OPB-08	37	37	100

Table 6. Representation data of distance matrix obtained by pair wise distance values

	G.cambogia	*G.mangostana*	*G.morella*	*G.indica*	*G.livingstonei*	*G.xanthochymus*
G.cambogia	0	0.763	0.662	0.65	0.486	0.794
G.mangostana		0	0.653	0.663	0.603	0.714
G.morella			0	0.670	0.576	0.820
G.indica				0	0.59	0.695
G.livingstonei					0	0.674
G.xanthochymus						0

representation.[25] The value here is at 80% and is a good score for a dendrogram. The dendrogram in figure 4indicates that the species are divided into 2 clusters: one that is solely *G. xanthochymus* and all other species in the other cluster with a genetic distance of 37%. Within the cluster with all other species, *G. cambogia* and G. livingstonei are most similar to each other with a 24% dissimilarity index. Both the species produce hydroxy citric acid and guttiferones as their main chemical component in fruits.[26,27] This is an indictator for the phenotypic relation. *G. morella* and *G. indica* have a 30% and 31% dissimilarity index to both G. cambogia and G livingstonei respectively. Both the plants have bright red fruits and are phenotypically similar. This is attributed to the presence of anthocyanin pigments in them. The oil from both the plants are utilised in cosmetic industries.[28] *G. mangostana* is the least similar in the group with a dissimilarity index of 33.5% in the group. This variety is introduced to India and the dendrogram justifies the same. The research on the origin of the Malaysian varieties of G. mangostana says this species may be the first in origins among the *Garcinia* varieties.[29]

G. xanthochymus is the most dissimilar with a score of 37% and is placed by itself in the dendrogram. The phenotypic characters of this plant are very different to the group in terms of fruit and leaf size. The chemical composition of these fruits is exclusive and includes xanthochymols.[30] The dendrogram representation outlines the relationships between and among these plant species. *G. cambogia* and *G. livingstonei*are closely related. *G. morella. G. indica*follows the cluster in relationship. Similar relation is corelated in the species of watermelons and disease resistance varieties is grouped

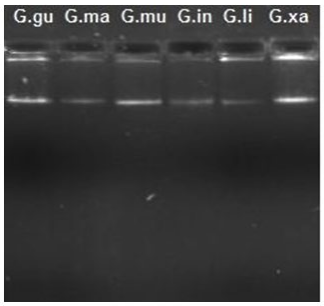

Fig. 1. DNA bands after column purification

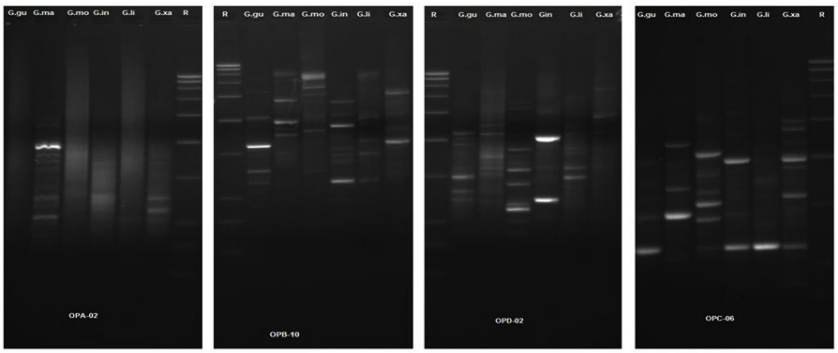

Fig. 2. RAPD profile generated by the PCR reactions from Primers-0PA-02; OPB-10;OPD-2;OPC-6

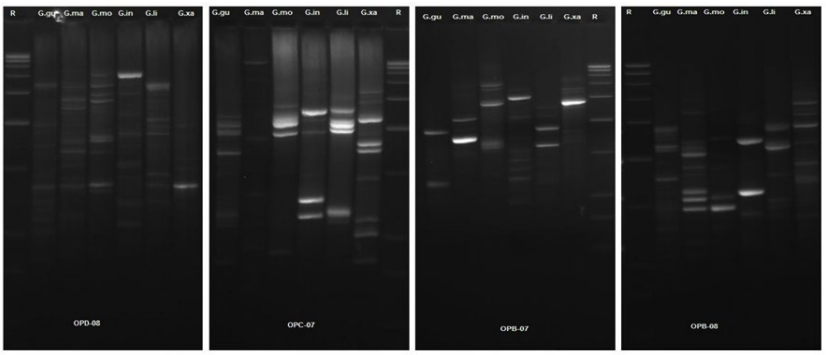

Fig. 3. RAPD profile generated by the PCR reactions from Primers:0PD-08; OPC-7; OPB-7; OPB-8

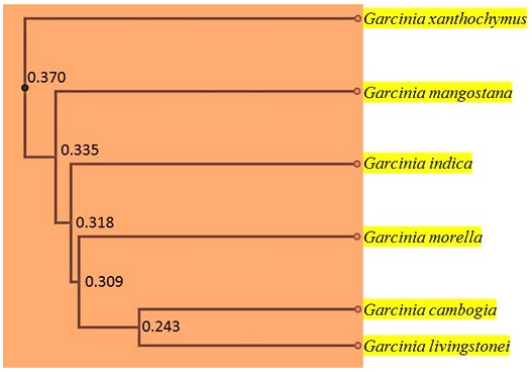

Fig. 4. UPGMA Dendrogram representation of relationship among various populations of *Garcinia* species

together.[31]In the current investigation the species of *Garcinia* is grouped together with relation to its phenotypic correlation. Chemical constituents and morphological characters are taken into consideration. The primers utilised in the study can be used to analyse the relationship amongclones in species. Similar work has been established in Popular and willow clones.[32] The study justifies *G. xanthochymus* is least similar to the group and is represented by the fact its phenotypically different in characters. The genetic distances can be correlated with the phenotype and this is the first reference towards the species in Garcinia.

CONCLUSION

The genetic distances derived among the species have suggested a preliminary relationship data among *Garcinia*. The CP value validates the accuracy of the analysis. With 98% of polymorphism in these markers, the sequences of the primers can be utilised for generating specific markers for future work. Genetic diversity study has shown relatedness to the phenotypic characterisation. The species being economically important, further investigation at the marker level can boost breeding and conservation programmes of these tree species. This research can be expanded to plausible gene tags for these species. The genetic diversity studies can be expanded to the *Garcinia* varieties in India.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support for the research work from the University grant commission under the minor research grant for college teachers.

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