Dissecting genetic diversity in *Garcinia xanthochymus* using ISSR and RAPD markers

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Abstract:

Western Ghats of India is one of the mega diversity centres in the world. There are 15 species of *Garcinia* reported of which many are endemic in nature. The genetic diversity in one of the same endemic species *Garcinia xanthochymus* was evaluated using ISSR and RAPD markers. Total of 15 individual plants from each location were analyzed. Six populations of *G. xanthochymus* were collected and screened using 16 ISSR and 10 RAPD markers. ISSR primers generated 80 bands of which 17 were polymorphic (21.25 % polymorphism) and RAPD primers produced 51 total bands of which 18 were polymorphic (35.29 % polymorphism). The ISSR and RAPD cluster analysis grouped them into two groups. The detected polymorphism is enough at species level and therefore the RAPD and ISSR can be used as efficient markers for genetic relatedness assessment in *G. xanthochymus.* The UPGMA and PCoA analysis distantly grouped these populations in two groups. The genetic diversity within the population is important source for evolution of the genera. *G. xanthochymus* has distinct populations in different geographical area.

Key words: Genetic diversity, Genetic improvement, ISSR, Polymorphism, RAPD.

Introduction:

The genus *Garcinia* L. belongs to the family Clusiaceae (Guttiferae). This family consists of approximately 200 species throughout the world, among which 36 species occur in India. The Western Ghats region is considered as a secondary center of origin for *Garcinia* species, where six species are endemic to Western Ghats only (Abraham et al. 2006). As a genus, *Garcinia* contains many species which include *Garcinia indica*, *Garcinia gummigutta*, *Garcinia hombroniana* and *Garcinia xanthochymus* that provide important resources in this region.
G. xanthochymus is distributed across the states of Maharashtra, Goa, Karnataka, Tamilnadu and Kerala. It is cultivated extensively in Southeast Asia where the fruit is used for preserves, jams, and curries. The dried fruit sap is called gamboge and provides a dye that is used in watercolor paints. Fruits are anthelmintic and cardiotonic; improves appetite. Ripe fruit is tonic, invigorating and alexipharmac; good in heart trouble and biliousness. Generally sherbat made with "Amsul" (sun-dried slices of the fruit), with a little rock-salt, pepper, cumin, ginger and sugar, is administered in bilious conditions (Yusuf et al. 1994). Xanthochymol present in the fruit is

antibacterial against *Streptococcus faecallis* and *Klebsiella pneumoniae*; the action is better than tetracycline (Asolkar et al. 1992). A new prenylated xanthone, 1,3,5,6-tetrahydroxy-4,7,8-tri(3-methyl-2-butenyl) xanthone , was isolated from the wood of *G. xanthochymus* together with a known xanthone, garcinia xanthone . Their structures were determined by spectroscopic analysis. There are certain reports on the production of antibacterial silver nanoparticles using the endophytic bactrerium *Bacillus cereus* isolated from *G. xanthochymus* (Sunkar and Nachiyar; 2012). However, the information on the genetic diversity of *G. xanthochymus* is not yet reported.

DNA fingerprinting is used to visualize DNA polymorphisms between samples. These fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relationship between samples. Fingerprints are also used as the source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and or genetic loci. Many DNA fingerprinting techniques have been developed in the past few years.

The genetic assessment of plant populations and species, for taxonomic, evolutionary, and ecological research, has been immensely assisted from the advancement of a range of molecular marker systems. Even though every molecular marker system is derived from distinctive principles, yet their function is to expose the genome-wide variability. Molecular markers offer a great advantage over the morphological and biochemical markers in species identification, phylogenetic studies and in mapping the genetic linkage (Suh et al. 2011). Combined approach of both morphological and molecular markers was also carried out to identify and characterize the species. Different molecular markers have been used for the genetic diversity studies in different *Garcinia* species. The Phylogenetic analysis of some plants in the genus *Garcinia* using Internal Transcribed Spacer (ITS) sequence data and AFLP analysis have been studied by Chinawat Yapwattanaphun (2003). Molecular investigations using ITS were carried out for identifying the origin of Mangosteen (Nazre, 2014). Mansyah et al. (2013) evaluated the genetic diversity among and within *G. mangostana* L. (Mangosteen) trees. Polymorphism in *G. indica* was assessed by the morphological and molecular markers. Various populations collected from Maharashtra screened with molecular markers, showed very less polymorphism (Thatte et al. 2012).

The significance of the germplasm is recognized only when it is characterized (Rao et al. 2002). Hence, the characterization of *G. xanthochymus* is necessary for its conservation and maintenance. The plants used in this study for characterization of *G. xanthochyms* were collected from different location of Western Ghats from Dapoli Maharashtra, ICAR Goa, Kadakeri, Siddhapur, Karnataka, GKVK Bangalore, Karnataka and NBPGR Thrissur, Kerala. Till date there are no reports based on the molecular markers for the genetic diversity for *G. xanthochymus.* Molecular markers are helpful in characterization of genetic divergence amongst several cultivars or populations of *G. xanthochymus* for the evaluation of genetic fidelity, identifying genes of commercial and agronomic interests, and enhancement via genetic transformation system. The aim of the present study was to fingerprint and explore the genetic relationship among the various populations of *G. xanthochymus* using ISSR and RAPD molecular markers

Material & Methods:

Collection of Plant material: Fruits (Figure 1) and Seeds of *G. xanthochymus* were collected from various regions, Dapoli, (Maharashtra state), Indian Counsel of Agriculture and Research (Goa), Kadakeri, Siddhapur (Karnataka), Gandhi Krishi Vidyan Kendra, Bangalore (Karnataka) and National Bureau for Plant Germplasm Resources, Thrissur (NBPGR, Kerala) and planted in Khalsa college garden. The GPS locations and details of collection sites of samples are shown in Table 1 and Figure 2. Young leaves of *G. xanthochymus* were collected just prior to extraction of DNA from Khalsa college garden.

Figure 1: Fruits of *G.xanthochymus*

Figure 2: Map showing collection sites of *G.xanthochymus*

Table 1: List of the *G. xanthochymus* populations used in present study

Accession No.	Location/Origin	GPS location	Sea Level
Population 1	Dapoli, Maharashtra	17^0 45' 13.65" N 73^0 10' 56.09" E	182m
Population 2	GKVK Bangalore, Karnataka	13^0 4' 49.69" N 77^0 34' 36.37" E	951m
Population 3	ICAR Complex Goa	$15^0$30'52" N $73^0$55'01" E	58m
Population 4	Kadakeri, Siddhapur, Karnataka	14^0 20' 27.3149" N 74^0 53' 20.8465" E	564m
Population 5 IC 552541-2	NBPGR, Thrissur, Kerala	10^0 33' 5.32" N 76^0 16' 30.60" E	33m
Population 6 IC 326809-1	NBPGR, Thrissur Kerala	10^0 33' 5.17" N 76^0 16' 30.63" E	25m

The DNA was isolated from six populations of *G. xanthochymus*. About 3 grams of fresh leaflets were ground for DNA extraction. Total DNA was extracted and purified according to the slightly modified CTAB protocol (Doyle and Doyle 1987). Purified DNA concentrations were determined with electrophoresis on 0.8% agarose gel, stained with 0.1 µg EtBr (ethidium bromide) and visualized using ultraviolet (UV) transilluminator. DNA was then used for PCR-amplification by using 16 ISSR and 10 RAPD primers obtained from the Eurofins.

Table 2: ISSR primers useful for the amplification of the six populations of *G. xanthochymus*

Primer	Sequence from 5'-3'	Tm °C
Primer A1	ACACACACACACACACTT	51°C
Primer A3	AGAGAGAGAGAGAGAGT	51°C
Primer A4	AGAGAGAGAGAGAGAGAA	51°C
Primer A5	AGAGAGAGAGAGAGAGTA	51°C
Primer A6	AGAGAGAGAGAGAGAGTT	51°C
17899B	CACACACACACAGG	49°C
ISSR14	GGAGTGGTGGTGGTG	53°C
ISSR11	AGAGAGAGAGAGAGAGGT	53°C
Primer 3c	CACACACACACACACAAT	53°C
Primer 4c	CACACACACACACACAAC	53°C
Primer 5c	CACACACACACACACAGT	53°C
Primer 873c	GACAGACAGACAGACA	49°C
Primer M10	CACACACACACAAGG	49°C

Table 3: RAPD primers useful for the amplification of the six populations of *G. xanthochymus*

Primer	Sequence 5'-3'	Tm °C
RP 1	AAAGCTGCGG	32°C
RP 2	AACGCGTCGG	34°C
RP 3	AAGCGACCTG	32°C
RP 4	AATCGCGCTG	32°C
RP 5	AATCGGGCTG	32°C
RP 6	ACACACGCTG	32°C
RP 7	ACATCGCCCA	32°C
RP 8	ACCACCCACC	34°C
RP 9	ACCGCCTATG	32°C
RP 10	ACGATGAGCG	32°C

Note: Tm: Melting temperature

PCR Reaction mixture: The chemicals were obtained from Thermo Fischer. Reactions were carried out in a total volume of 25 µl consisting of 100 ng of template DNA, 1X Taq. Buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.2mM dNTPs, and 0.5µM Primer. The total volume was made up using nuclease free water. Further, the reaction mixture was kept in the thermal cycler for amplification.

Amplification was performed under the following conditions:

5 min at 95°C for 1 cycle, followed by 1 min at 94°C, 1 min at annealing temperature (depending on the primer used), 1 min at 72°C for 45 cycles, and 7 min at 72°C for final extension. PCR products were separated on 2% agarose gel stained with 0.1 µg/ml of ethidium bromide using 1X TAE buffer solution, and visualized under UV light.

Data Analysis:

The amplified product of PCR was scored as presence of band (1) or absence of band (0) for the 90 population of *G.xanthochymus*. The relationship among the population was calculated through Jaccard coefficient. This analysis was performed using the un-weighted pair group method with arithmetic means (UPGMA) algorithm computed by DendroUPGMA(Garcia-Vallvé.S and Puigbo.P,2002) and PAST version 3.12 (PAleontological Statistics).The PCoA analysis was carried out to visualize genetic diversity among population. The analysis was carried out using GenAlEx 6.5 (Peakall and Smouse, 2012) .

Results:

ISSR analysis

Sixteen primers produced a total of 80 bands among the *G. xanthochymus* genotypes. The size of the amplified products ranged from 201 bp to 2000 bp. The number of scorable bands produced per primer ranged from 3 to 7. The total number of polymorphic bands and the percentage of polymorphism were 17 and 21.25 respectively (Table 4) (Figure 3 and 4).

ISSR A5 showed only one polymorphic band of 1300 bp, present in population of Dapoli, Bangalore and Goa (Figure 3) while in other three populations from Siddhapur, Thrissur 1(IC 552541-2) and Thrissur 2 (IC 326809-1) bands were absent. The ISSR trail of primer 3C showed three polymorphic bands in five populations whereas population of Thrissur1 showed absence of band in 900bp, 1500bp and 1600bp region (Figure 4).

Dendrogram was constructed from the data obtained from ISSR analysis. There were 2 clusters formed where first cluster consisted of population of Dapoli and Thrissur 2 while the rest of population lied in second cluster (Figure 7 A)

RAPD analysis

The total number of bands observed among *G. xanthochymus* based on RAPD analysis was 51. The number of scorable bands produced per primer ranged from 3 to7 and size of the products ranged from 300 bp to 1700 bp. The total number of polymorphic bands and the percentage of polymorphism were 18 and 35.29 respectively (Table 2) (Figure 5 and Figure 6).

Amplification produced by RP 4 primers, 1000 bp of band showing polymorphism in *G. xanthochymus*. It was produced in Dapoli and Siddhapur populations.

A comparison of the amplification pattern produced by RP 10 (Figure 6) showed six polymorphic bands of size 280 bp, 500 bp, 700bp, 800 bp and 900 bp in the six populations. 280 bp band was present in Dapoli, Thrissur 1 and Thrissur 2, 500 bp band was present in three populations Bangalore, Goa and Siddhapur respectively. 700 bp band was present in Bangalore, Goa, Siddhapur and Thrissur 2 populations while 800 and 900 bp bands were observed only in Bangalore, Goa and Siddhapur populations.

Dendrogram was constructed from the data obtained from RAPD analysis. There were 2 clusters formed where first cluster consisted of populations of Dapoli, Thrissur1 and Thrissur 2 while the rest of population lied in second cluster (Figure 7 B)

Table 4: Polymorphism observed in different populations of *G. xanthochymus* using molecular markers

Markers	No. of primers	Total no. of bands	No. of polymorphic bands	% of polymorphism
RAPD	10	51	18	35.29%
ISSR	16	80	17	21.25%

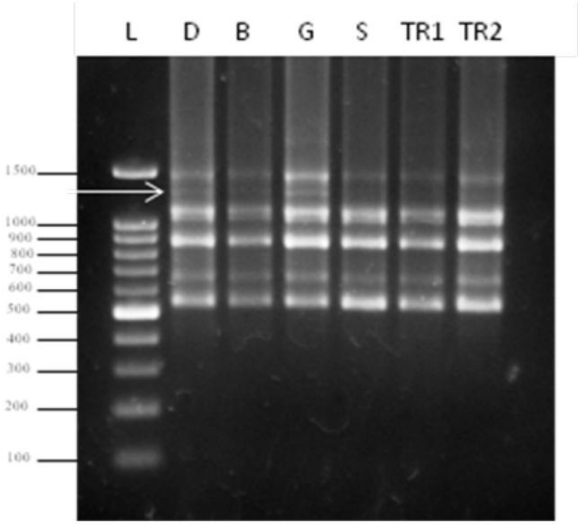


Figure 3: ISSR fingerprints of six populations of *G. xanthochymus* generated with primer A5

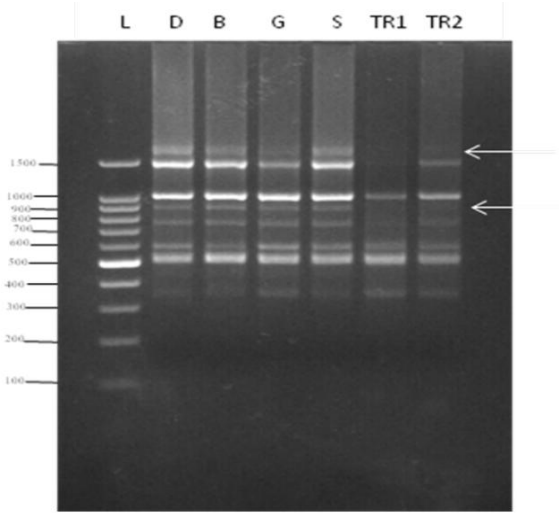


Figure 4: ISSR fingerprints of six populations of *G. xanthochymus* generated with primer 3C

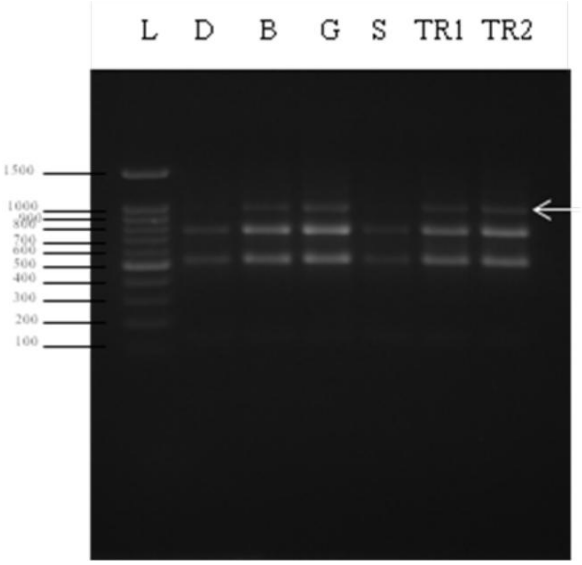


Figure 5: RAPD fingerprints of six populations of *G. xanthochymus* generated with primer RP4

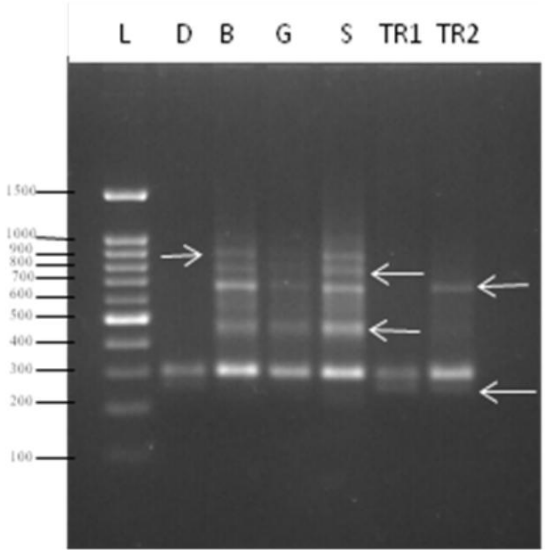
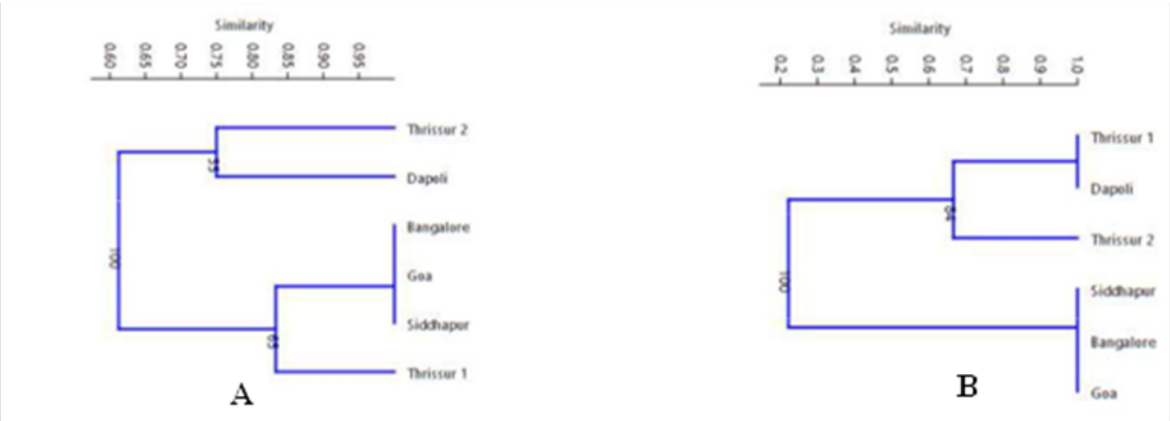
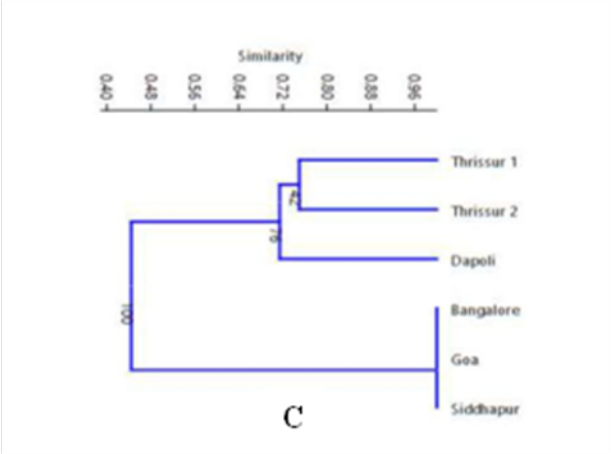


Figure 6: RAPD fingerprints of six populations of *G. xanthochymus* generated with primer RP 10

Note: Loading Sequence: Lane 1-Ladder (L), 2-Dapoli (D), 3- Bangalore (B), 4- Goa (G), 5-Siddhapur (S), 6-Thrissur 1 (TR 1), 7- Thrissur 2 (TR 2)

Figure 7: UPGMA dendrogram based on the similarity matrix of *G. xanthochymus* obtained with the Jaccard coefficient for the data A) 16 ISSR primers B) 10 RAPD primers C) 26 RAPD+ISSR primers



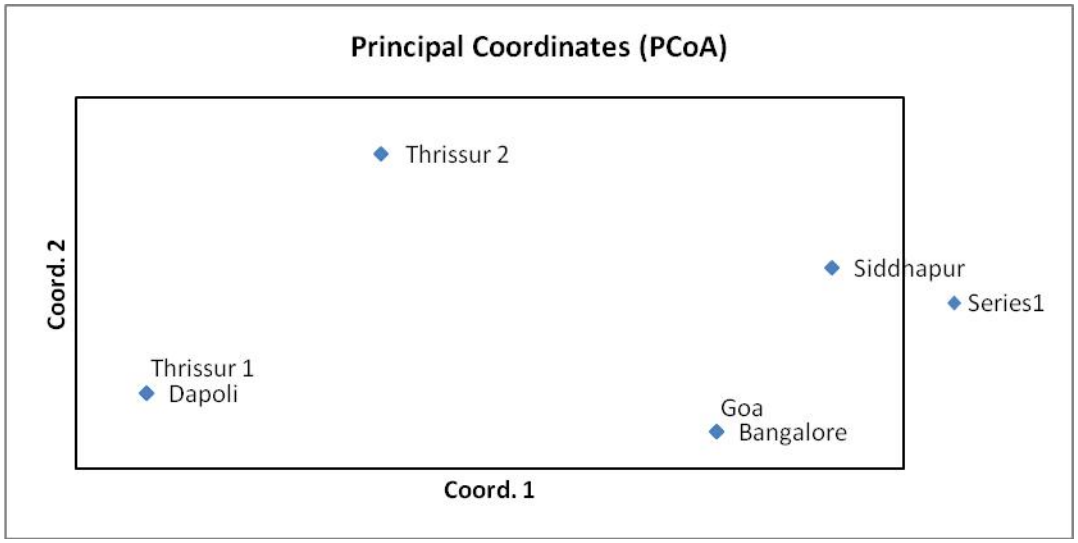


Cluster analysis

126 bands were formed by analyzing the RAPD and ISSR techniques together which were used to construct a dendrogram based on Jaccard similarity coefficient. Cluster analysis performed using RAPD and ISSR generated a dendrogram that separated the genotypes into two distinct clusters (Figure 7 C). The first cluster included genotypes of Dapoli, Thrissur1 and Thrissur 2. It is divided into subcluster 1(a) consisting of Dapoli and Thrissur1 and Thrissur2 stands alone. The second cluster included genotypes of Bangalore, Goa and Siddhapur. It is divided into subcluster 2(a) consisting Bangalore and Goa, and Siddhapur stands alone in cluster.

The Principal Coordinate Analysis performed with the complete set of molecular data (RAPD+ISSR) for 6 *G. xanthochymus* genotypes, accounted for 54.41%, 36.00%,9.58% of variation as explained by the first three axes. Principal Coordinate 1 separates the genotypes belonging to cluster 2 from the cluster 1 (Figure 8)

Figure 8 : Projection of the genotypes onto the plane defined by the Principal Coordinates 1 and 2, from the data of 26 markers (RAPD, ISSR)



Discussion:

The *Garcinia* species in India show rich source of biodiversity (Abraham et al 2006) and their conservation and utilization requires further characterization at inter and intra species level. Comparative studies on various *Garcinia* species involving RAPD, ISSR and SSR markers were successfully used by various researchers throughout the world (Mansyah et al, 2010; Qosim et al., 2011,; Mohan et al, 2012; Thatte et al,2012;Tharachand et al, 2015).

The discriminative power of DNA markers used as tool to characterize the *Garcinia* species is essential because they can be used to assess the genetic diversity among *Garcinia* species. During the characterization of *G. xanthochymus* from Western Ghats, the discriminative power of RAPD and ISSR markers systems were evaluated. Among the two marker system employed 10 RAPD primers produced a total of 51 bands whereas 16 ISSR primers produced 80 bands. The level of polymorphism revealed by RAPD (35.29%) is higher than ISSR (21.25%). Thatte et al. (2012) also reported very less diversity in *G. indica* using morphological and molecular markers (RAPD and ISSR) collected from Maharashtra. The highest diversity within that population was 19.93%.

ISSR and RAPD markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (Kumar et al. 2014). They also may have potential for analyzing biogeographic patterns among populations of a single plant species. In this study, we have shown that these markers revealed genetic variation among geographically separated samples of *G. xanthochymus* collected from different locations of Western Ghats. The results obtained in this study showed ISSR and RAPD markers measured sufficient polymorphism in *G. xanthochymus* and can be used in germplasm characterization and fingerprinting purposes.

Cluster analysis was carried out on two sets of marker profiling data based on combination of RAPD and ISSR. Cluster 1 is a heterogenous in terms of geographical location. The Thrissur1 and Thrissur2 genotypes were collected from Thrissur, Kerala which are placed along with Dapoli genotypes indicating that they share some genetic similarity.

Cluster 2 can also be called as heterogenous in terms of geographical location. Bangalore, Goa and Siddhapur populations were shown there can be a possible relationship among the three genotypes. This result was comparable to the genetic variation showed by UPGMA dendrogram. The same comparative results were found in UPGMA dendrogram and Principle Coordinate Analysis of *G. gummigutta* (Tharachand et al 2015). The Principle Coordinate Analysis (PCoA) plot generated by the RAPD primers showed the relationship of the populations and genetic variabilities in *G. gummigutta*.

This study may be indicative that due to geographical and topological difference the genotypes grouped into 2 clusters would have gone a significant variation that can be seen from their molecular characteristics.

Molecular phylogenetic studies of *G. xanthochymus* have shown vast genetic diversity using RAPD and ISSR markers. A better understanding of genetic diversity and its distribution is wanted for their conservation strategy. This study revealed high level of genetic diversity among six populations of *G. xanthochymus* collected from various locations. Geographical conditions

and surrounding environment made an impact on plant species which is evidenced by this study. Though the *G. xanthochymus* populations were not showing much diversity in their morphology, but showing enough genetic diversity at species level to discriminate the populations. This study will help and encourage to other researchers and plant breeder to understand the molecular diversity of *G. xanthochymus*.

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