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Genetic Diversity and Fingerprinting of *Garcinia indica*

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Abstract:
Genomic DNA of 19 samples from Garcinia indica was isolated. The average number of alleles across all microsatellite loci stood at 18, whereas, the number of different alleles across microsatellite loci ranged between 6 and 16 with an average of 11.727. (Table1). The microsatellite marker loci ITBTGI19 and ITBTGI21 recorded highest number of different alleles (16). The overall mean of I was 2.296, The grand mean of expected heterozygosity (He) was 0.877. (Table1). Among all the loci. The mean heterozygosity values across all loci ranged between 0.756 (ITBTGI9) to 0.956 (ITBTGI19). The pair-wise genetic distance among the accessions of G. indica, the values ranged from a minimum of 56 to a maximum of 88 between different accessions the accession GI_MDG_4 recorded genetic distance of 88 with most of the other accessions Table2. The microsatellite ITBTGI1 recorded maximum allelic size of 1000 for the accession GI-KAN 3 (Table3).

Keywords: Garcinia indica, Genetic diversity, DNA, Marker

INTRODUCTION

Genus *Garcinia*, a largest member of the family Clusiaceae (Guttiferae), which comprises more than 250 species, are widely distributed across the tropical world, among them, 35 species are found in India, of which six are endemic to the evergreen forests of *Western Ghats* (Peter and Abraham, 2007). The genus *Garcinia* is native to the South East Asia. In India, *Garcinia* species propagate widely in a semi-wild state, in Maharashtra 'Konkan' area, Goa, coastal areas of Karnataka and Kerala, Assam evergreen forests, Khasi, Jantia hills, West Bengal and Gujarat.

Garcinia species are evergreen trees and shrubs which thrive well in high rainfall areas of the tropics. The trees are dioecious and hence are cross-pollinated. The fruits of *Garcinia* species show the anti-obesity property because of presence of the compound called (-)-Hydroxycitric acid (HCA), which made these species popular in the international market. The most popular species of the genus *Garcinia* is *G. mangostana*, which is commonly known as mangosteen and has been named as 'queen of tropical fruits' for its unique pleasant taste and visual appearance of a crown-like structure (Chinavat and Subadrabuddhe, 2004). The seeds and pericarps of the fruit have a long history of use in the traditional medicinal practices of the region, and beverages containing mangosteen pulp and pericarps are sold worldwide as nutritional supplements. The fruits of *Garcinia* species have an anti-obesity compound called (-)-Hydroxycitric acid (HCA), which made

these species popular in the international market. Other phytochemicals present in the species are isoprenylated xanthones, a class of secondary metabolites with multiple reports of biological effects, such as antioxidant, pro-apoptotic, anti-proliferative, antinociceptive, anti-inflammatory, neuroprotective, hypoglycemic and anti-obesity.

The center of diversity of Garcinia species is the Malaysian region, with some species reaching India and the Micronesian islands and also extending to tropical Africa and the Neotropics (Rogers and Sweeney 2007; Stevens, 2007; Jones, 1980; Sharma et *al.*, 2013; Nimanthika and Kaththriarchi, 2010). Genetic diversity of a particular species at different levels are analyzed by various methods which include; morphological characterization of the individuals in different populations of the species, biochemical analysis using allozymes and use of molecular markers. Among these methods, using molecular markers such as DNA markers is considered an efficient way to assess genetic diversity (Mondini *et al.,* 2009). Molecular markers overcome the limitations of morphological characterization by being stable and unaffected by environment and that of, biochemical analysis by being abundant in the majority of the species. Thus, using the suitable molecular DNA based markers for studying genetic diversity gives us a better understanding of the organism, its genetic makeup and its diversity at different levels of the ecosystem. Simple Sequence Repeats (SSR) or microsatellite markers are a short stretch of nucleotides as sets of two to six base-pairs repeating sequences which are grouped as DNA markers found within the eukaryotic genome. SSR markers are widely used in animal and plant species for their advantages in being co-dominant, showing high allelic diversity and abundance. Their high reproducibility and ease of accessing the SSR size variation through PCR amplification along with flanking primers make them popular genetic markers to be used in diversity studies (Qosim *et al*., 2011).

MATERIAL AND METHOD

Genomic DNA of 19 samples from *Garcinia indica* was isolated. The protocol for genomic DNA isolation given by Doyle and Doyle (1987) was followed up with a few modifications.

Procedure

1. About 1 g of fresh leaf tissue from each sample was chopped and mixed with 1 ml of CTAB extraction buffer in 2 ml centrifuge tubes. Samples were crushed using Tissuelyser –II (Qiagen). Frequency used was 25/sec for 10 minutes.
2. The samples were kept in water bath for 30- 45 minutes at 65˚C.
3. The incubated samples were centrifuged at 10,000 rpm for 10 minutes at room temperature.
4. The supernatants were transferred in to fresh tubes after discarding the leaf debris. 500 µl of Chloroform-Isoamyl alcohol (24: 1) was added to each sample and then centrifuged at 10,000 rpm for 10 minutes at room temperature.
5. The above step was repeated once or twice until a clear bilayer of supernatant and debris was obtained.
6. The supernatants were collected in fresh tubes. For 500 µl of supernatant 300 µl of chilled isopropanol was added and mixed well and stored at -20° C for overnight.
7. The samples were centrifuged for 10 minutes at 10000 rpm to obtain a pellet containing genomic DNA.
8. The supernatant was drained from the tubes and pellets were washed by centrifuging with 500 µl of 70 per cent ethanol at 10000 rpm for 10 minutes.

9. The pellets were dried at room temperature for complete evaporation of ethanol. About 40 µl of TE buffer (Tris HCl: EDTA in 10: 1) was added to each DNA sample for dilution of DNA.

PCR Amplification of DNA Samples Using SSR Markers

PCR amplification of genomic DNA of *Garcinia indica* was carried out using SSR primer pairs synthesized at CBR, Department of Biotechnology and Crop Improvement, College of Horticulture, Bengaluru

Separation of Microsatellite Markers

Separation and visualization of PCR products was done on agarose (2.5 per cent). Agarose was casted in 2.5 per cent gels in TAE buffer (1X). Slabs gels were casted in a horizontal gel frame (Hoefer HE99X 18 x 30 cm, Amersham Bioscience Pvt. Ltd. USA). Products were visualized by incorporating 1 µl (10 mg/ml) of Ethidium Bromide (ETBR) per 10 ml of gel solution and viewed in a gel documentation system (Syngene Pvt. Ltd.

Statistical Analysis

The gel pictures were scored for allele size using Gen Alexs 6.5 software algorithm. Genetic diversity parameters, *viz*., number of alleles (Na), number of effective alleles (Ne), gene diversity (He), observed heterozygosity (Ho), major allele frequency (MAF), polymorphic information content (PIC), Wright's inbreeding coefficient (F), and Shannon's information index (I), were estimated for the 25 SSR markers in *Garcinia indica* samples. Wherein, "Ho" is the actual amount of heterozygosity measured at a particular locus or population, and "He" is the proportion of heterozygosity expected for a particular locus or population undergoing random mating and acting within Hardy-Weinberg equilibrium. The decline in heterozygosity due to subdivision within a population was quantified using an index known as Wright's "F"-statistics (F) (Wright 1965). "PIC" and "I" are the marker in formativeness and discriminating power of the markers based on the allelic frequency of each locus. Statistical analysis was performed using the software packages GenAlex 6.5 (Peakall and Smouse 2012) and Power Marker 3.25 (Liu and Muse 2005). Locus wise F-statistics (F_{st}, F_{is}, and F_{it}) and level of gene flow ($Nm = (1/F_{st})-1)/4$) were estimated for the subpopulations to find out the efficiency of 25 DNA markers in genetic discrimination of using the soft- ware package Popgene (Yeh *et al.*, 1999).

RESULT

Polymorphism and Allelic Frequency

The polymorphism and allelic frequency of *G. indica* selected 19 accessions was assessed using a set of 22 microsatellite markers. The average number of alleles across all microsatellite loci stood at 18, whereas, the number of different alleles across microsatellite loci ranged between 6 and 16 with an average of 11.727 across the selected accessions of *G. indica*. The microsatellite marker loci ITBTGI19 and ITBTGI21 recorded highest number of different alleles (16) followed by marker loci ITBTGI5, ITBTGI7, ITBTGI15, ITBTGI16 and ITBTGI22 with 14 alleles and loci ITBTGI9and ITBTGI11recorded least number of different alleles (6). Number of effective numbers of alleles (Ne) recorded on an average of 9.116 across all the loci and maximum for the marker loci ITBTGI19 (14.440) followed by ITBTGI21 (13.370) and the marker loci ITBTGI9 recorded the minimum value of 3.767. Shannon's Information Index (I) was considered as a parameter for genetic variation among the microsatellite loci. The overall mean of I was 2.296. Among all the loci the grand mean of observed heterozygosity (Ho) was 0. The grand mean of expected heterozygosity (He) was 0.877. The mean heterozygosity values across all loci ranged between 0.756 (ITBTGI9) to 0.956

(ITBTGI19). The allelic frequencies of microsatellite loci and their grand mean is presented in Table 1.

The pair-wise genetic distance among the accessions of *G. indica*, the values ranged from a minimum of 56 to a maximum of 88 between different accessions. The genetic distance of 88 was exhibited among many *G. indica* accessions.

The accession GI_MDG_4 recorded genetic distance of 88 with most of the other accessions, while the least genetic distance of 56 was exhibited between GI_KOK_1 and GI_KAR_15. The pair-wise genetic distance for all the accessions of *G. indica* are presented in Table2. The allele frequencies across loci were assessed with Graph over Loci for Codominant Data for *G. indica* accessions, where two markers- ITBTGI8 and ITBTGI10 showed higher allelic frequencies followed by markers ITBTGI2 and ITBTGI1 and the markers with low allelic frequency were; ITBTGI4, ITBTGI18 and ITBTGI20 among the 22 markers across loci. The Graph over Loci for Codominant Data for *G. indica* accessions is presented in Figure.1.

Principal Coordinate Analysis

The Principal Coordinate Analysis (PCA) was carried out to understand the relationships between the sampled accessions of *G. indica* based on genetic distance and is represented in Figure.2.

Both upper and lower quadrants on the right represented *G. indica* accessions sampled from Uttara Kannada and Dakshina Kannada whereas, the left upper quadrant consisted of one accession sampled from Udupi along with Uttara Kannada and Dakshina Kannada and the lower left quadrant represented the one accession sampled from Chikmagalur along with Uttara Kannada and Dakshina Kannada.

Phylogenetic Analysis

The Neighbour Joining (NJ) phylogenetic tree constructed across 19 accessions of *G. indica* (Figure 3) in the present study revealed the existence of two major clusters - Cluster 1 and Cluster 2. The cluster 1 was further sub-divided into two sub-clusters (C1a and C1b).

Sub-cluster C1a consisted of accessions sampled from Uttara Kannada and Dakshina Kannada regions, whereas the sub-cluster C1b consisted of accessions sampled from Udupi, Uttara Kannada and Dakshina Kannada. On the other hand, the sub-cluster C2 consisted of accessions from Chikmagalur along with accessions from Uttara Kannada and Dakshina Kannada.

DNA Fingerprinting of *Garcinia indica* Accession

A set of twelve polymorphic microsatellite markers with reproducible amplification pattern were scored for DNA fingerprinting of 19 selected *G. indica* accessions.

The microsatellite ITBTGI1 recorded maximum allelic size of 1000 for the accession GI-KAN 3, whereas, the microsatellite marker ITBTGI12 exhibited least allelic size of 107 specific to the accession GI-KAN20. Three different microsatellites; ITBTGI4, ITBTGI16 and ITBTGI20 recorded different allelic sizes of 218, 184 and 220, respectively, for the accession GI-SIR 5. The allelic sizes of the PCR amplified products using these microsatellite markers for all accessions is presented in (Table.3).

Table 1: Heterozygosity, F statistics and polymorphism of *G. indica* by locus for codominant data

	N	Na	Ne	I	Ho	He	UHe	F
ITBTGI1	19	9	5.730	1.937	0.000	0.825	0.848	1.000
ITBTGI2	18	10	6.231	2.058	0.000	0.840	0.863	1.000
ITBTGI3	16	10	8.000	2.187	0.000	0.875	0.903	1.000
ITBTGI4	19	13	8.395	2.361	0.000	0.881	0.905	1.000
ITBTGI5	18	14	11.571	2.553	0.000	0.914	0.940	1.000
ITBTGI6	18	12	9.000	2.351	0.000	0.889	0.914	1.000
ITBTGI7	18	14	11.571	2.553	0.000	0.914	0.940	1.000
ITBTGI8	17	11	8.257	2.262	0.000	0.879	0.906	1.000
ITBTGI9	18	6	3.767	1.503	0.000	0.735	0.756	1.000
ITBTGI10	17	10	8.758	2.232	0.000	0.886	0.913	1.000
ITBTGI11	18	6	4.050	1.565	0.000	0.753	0.775	1.000
ITBTGI12	18	10	8.526	2.216	0.000	0.883	0.908	1.000
ITBTGI13	15	10	7.759	2.176	0.000	0.871	0.901	1.000
ITBTGI14	18	11	8.526	2.274	0.000	0.883	0.908	1.000
ITBTGI15	19	14	11.645	2.552	0.000	0.914	0.939	1.000
ITBTGI16	19	14	10.314	2.507	0.000	0.903	0.927	1.000
ITBTGI17	19	13	10.939	2.479	0.000	0.909	0.933	1.000
ITBTGI18	17	13	9.323	2.425	0.000	0.893	0.920	1.000
ITBTGI19	19	16	14.440	2.726	0.000	0.931	0.956	1.000
ITBTGI20	19	12	8.805	2.333	0.000	0.886	0.910	1.000
ITBTGI21	19	16	13.370	2.698	0.000	0.925	0.950	1.000
ITBTGI22	18	14	11.571	2.553	0.000	0.914	0.940	1.000
Mean	**18**	**11.72**	**9.116**	**2.296**	**0.000**	**0.877**	**0.902**	**1.000**
S.Em±	0.22	0.58	0.57	0.06	0.00	0.01	0.01	0.00

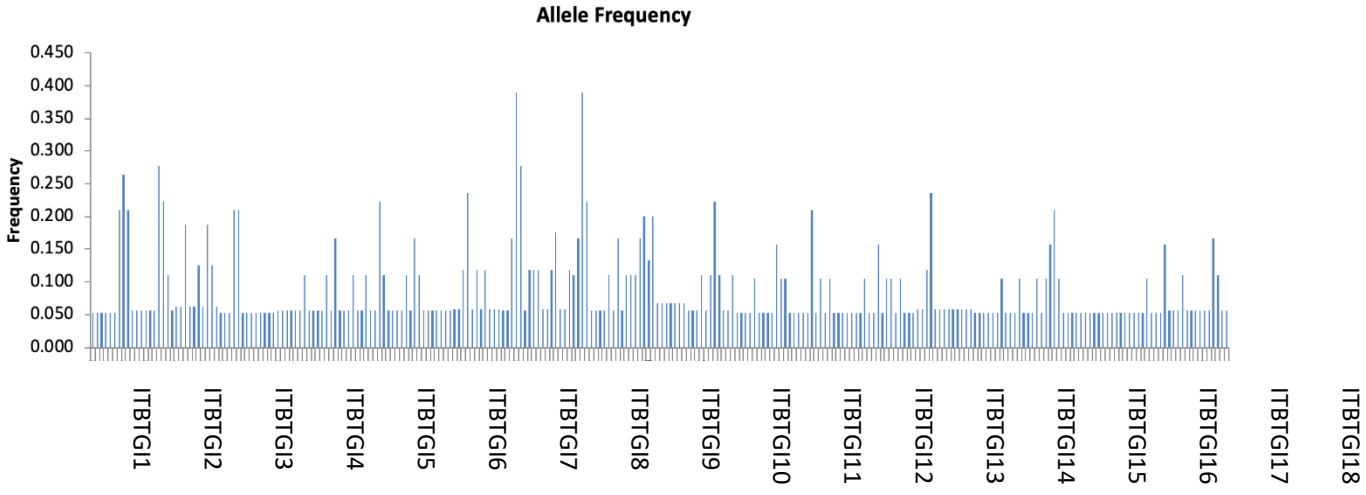

Figure 1: Allele Frequencies of *G. indica* by population with graph over loci for codominant data

Table 2: Euclidean distance of each genotype based on cluster analysis in *G. indica*

	GI_KAR_5	GI_KAR_9	GI_KAAZAR_10	GI_KAR_14	GI_KAN_13	GI_KAN_3	GI_KAN_4	GI_KAN_5	GI_KAN_6	GI_KAN_7	GI_KAN_20	GI_SIR_5	GI_KAR_15	GI_KOK_1	GI_KAN_12	GI_KAN_15	GI_SID_1	GI_PUT_1	GI_MDG_4
GI_KAR_5	0																		
GI_KAR_9	80	0																	
GI_KAR_10	76	60	0																
GI_KAR_14	76	72	76	0															
GI_KAN_13	80	80	80	84	0														
GI_KAN_3	84	88	68	84	80	0													
GI_KAN_4	80	88	84	80	76	64	0												
GI_KAN_5	88	84	84	84	84	68	72	0											
GI_KAN_6	76	84	84	84	80	88	80	84	0										
GI_KAN_7	80	80	88	80	80	84	76	80	80	0									
GI_KAN_20	84	84	88	88	88	88	84	84	76	72	0								
GI_SIR_5	80	80	84	84	88	80	84	80	84	80	76	0							
GI_KAR_15	88	84	84	84	72	84	84	80	88	80	88	72	0						
GI_KOK_1	88	84	84	88	80	80	84	80	88	84	88	84	56	0					
GI_KAN_12	84	80	88	80	84	88	80	88	80	84	84	84	84	72	0				
GI_KAN_15	88	76	84	80	84	80	88	84	84	88	80	88	80	72	64	0			
GI_SID_1	84	88	84	80	76	76	88	84	84	88	88	84	76	84	84	76	0		
GI_PUT_1	84	88	88	84	76	88	88	88	80	88	84	88	76	88	84	84	64	0	
GI_MDG_4	84	88	88	88	84	88	84	88	84	88	84	88	88	88	88	88	84	68	0

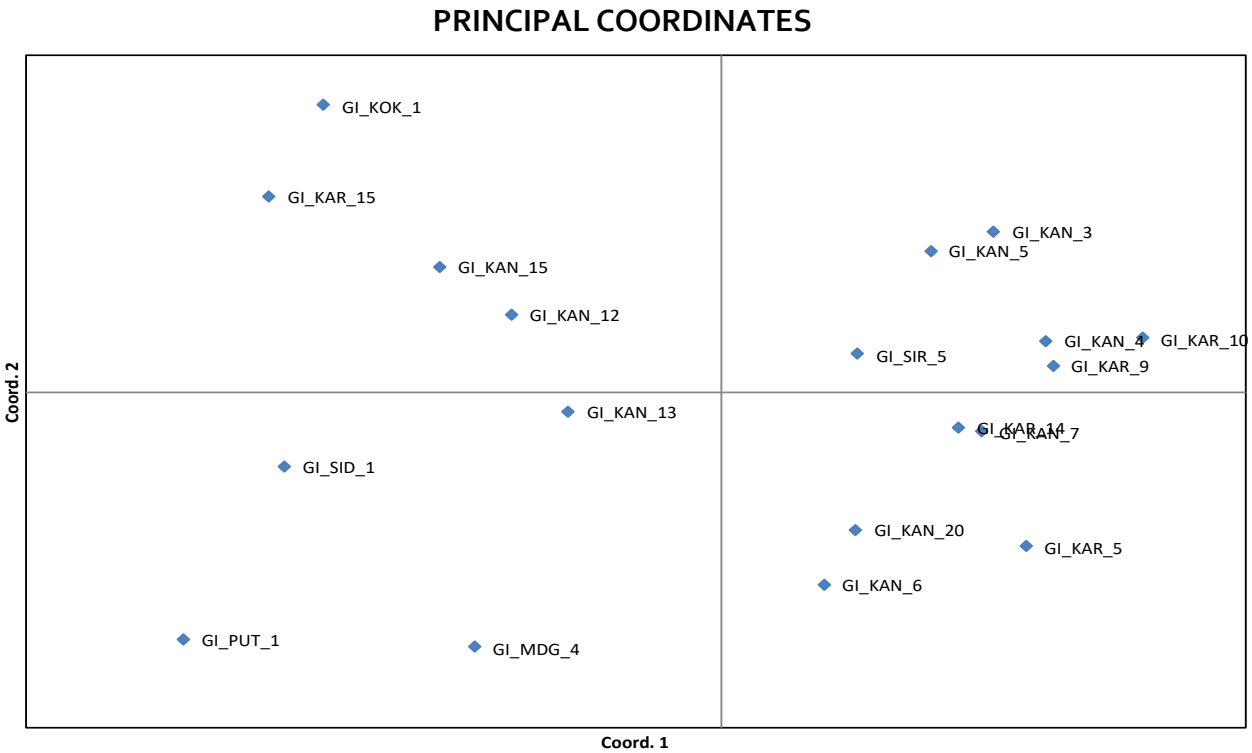

Figure 2: Principle coordinates analysis (PCA) using pair-wise genetic distance matrix of 19 G. indica accessio

Table 3: DNA Fingerprints for selected *G. indica* accessions

Sl. No.	Accession	Fingerprints		
1	GI- KAR 5	(ITBTGI26) 256		
2	G.I- KAR 9	(ITBTGI8) 409		
3	G.I- KAR 10	(ITBTGI8) 414		
4	G.I- KAR 14	(ITBTGI8) 392		
5	G.I- KAN 13	(ITBTGI8) 414		
6	G.I- KAN 3	(ITBTGI1) 1000		
7	G.I- KAN 4	(ITBTGI18) 211		
8	G.I- KAN 5	(ITBTGI16) 144		
9	G.I- KAN 6	(ITBTGI18) 144		
10	G.I- KAN 7	(ITBTGI18) 146		
11	G.I- KAN20	(ITBTGI12) 107		
12	G.I- SIR 5	(ITBTGI4) 218	(ITBTGI16) 184	(ITBTGI20) 220
13	G.I- KAR 15	(ITBTGI16) 165		
14	G.I- KOK 1	(ITBTGI16) 161	(ITBTGI16) 161	(ITBTGI20) 195
15	G.I- KAN 12	(ITBTGI18) 193	(ITBTG I20) 227	
16	G.I- KAN 15	(ITBTGI7) 219		
17	G.I- SID 1	(ITBTGI6) 300		
18	G.I- PUT 1	(ITBTGI22) 319		
19	G.I- MDG 4	(ITBTGI19) 204		

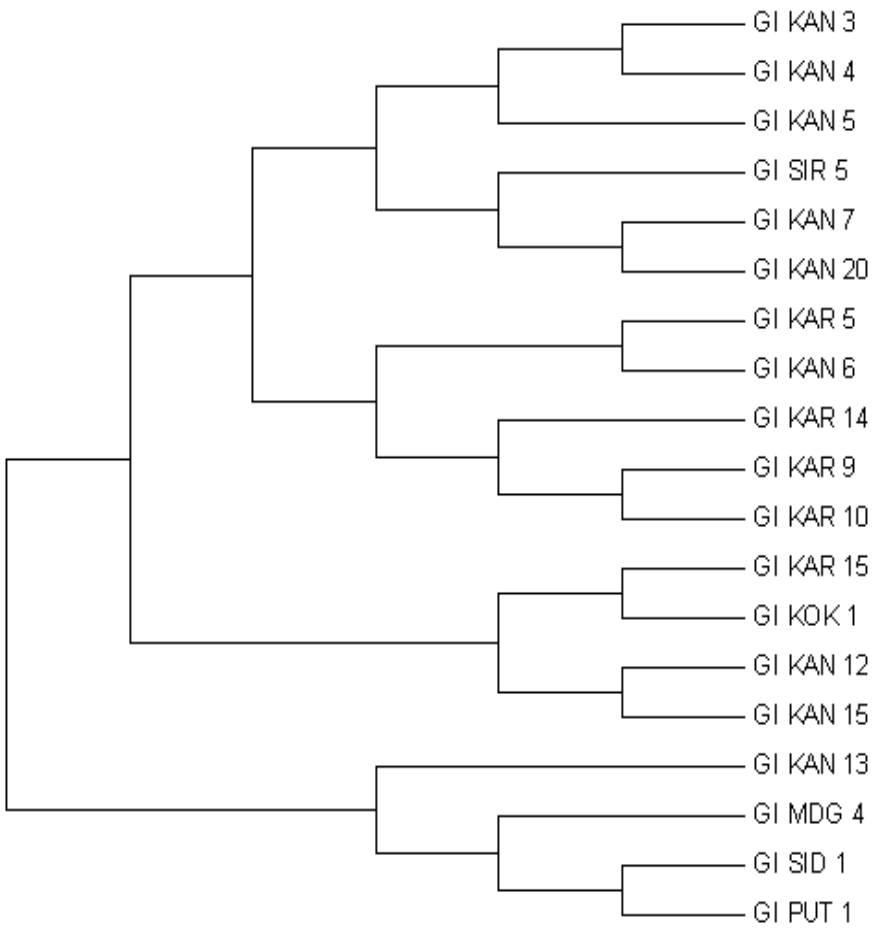

Figure.3: Neighbor Joining (NJ) tree constructed using MEGA V5 based on pair-wise genetic distance across *G. indica* accessions

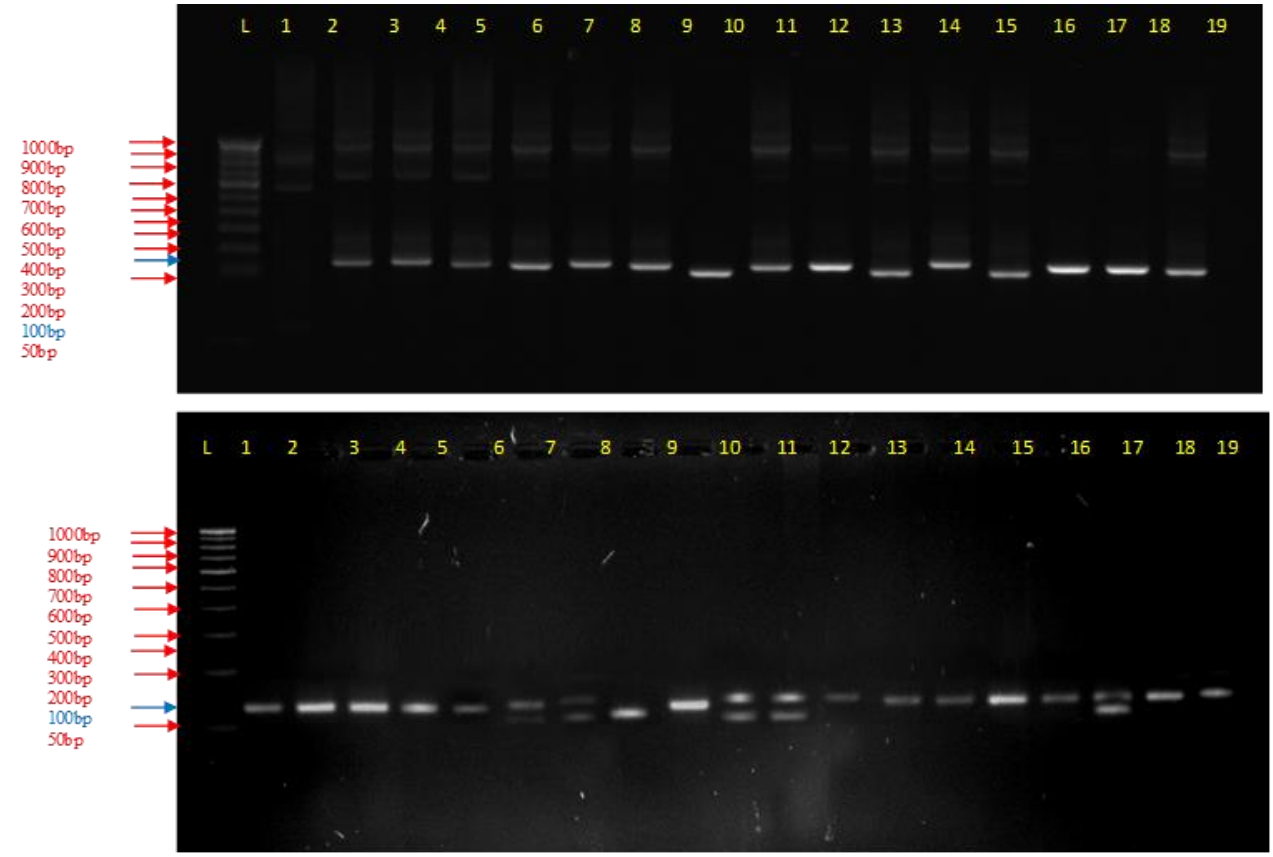

Plate 4: 8.PCR profile *G. indica* accessions using SSR marker. The number1 to 19 represent *G. indica* accessions and L represents ladder (100bp)

DISCUSSION

The genetic diversity of *Garcinia* species can be assessed by efficient techniques involving biochemical and molecular approaches. Among them, molecular markers have proven to be an efficient tool for elucidating genetic diversity present in the species. Several works of genetic diversity analysis in *Garcinia* species have used various molecular markers such as RAPD (Tharachand, 2015), ISSR (Mansyah *et al.*, 2010), AFLP (Sobir *et al.*, 2009), SSR (Qosim *et al.*, 2011), *etc*. which were efficient in various aspects of genetic diversity analysis as evidenced by the results of respective studies. Simple Sequence Repeats (SSR) or microsatellites are efficient genetic markers that are popular because of their co-dominant inheritance, high abundance, the enormous extent of allelic diversity, ease of assessing SSR allele size variation through PCR and high reproducibility (Mondini *et al.*, 2009). In the present, study SSR markers were used and results are discussed in the following sections.

Polymorphism and allelic frequency of *G. indica* accessions were assessed using 22 microsatellite markers. The average number of alleles across all microsatellite loci stood at 18, whereas the number of different alleles across microsatellite loci ranged between 6 and 16 with an average of 11. 272 across the accessions of *G. indica*. The loci GI_M19 and GI_21 recorded the highest number of different alleles (16) followed by loci GI_M5, GI_M7, GI_15, GI_16 and GI_22 with 14 alleles and loci GI_M9 and GI_M11 recorded the least number of different alleles (6). The number of Effective numbers of alleles (Ne) recorded an average of 9.116 across all the loci and maximum for the loci GI_M19 (14.440) followed by GI_M21 (13.370) and the loci GI_M9 recorded the minimal value of 3.767.

Shannon's Information Index (I) was considered as a parameter for genetic variation among the microsatellite loci. The overall mean of I was 2.296. Among all the loci the grand mean of observed heterozygosity (H0) was 0. The grand mean of expected heterozygosity (He) was 0.877. The mean heterozygosity values across all loci ranged between 0.756 (GI_M9) to 0.956 (GI_M19). The allelic frequencies of microsatellite loci and their grand mean are comparable to previous reports by Roy *et al*. (2015) and Ravishankar *et al.* (2015) in *G. indica*. The pair-wise genetic distance among the accessions of *G. indica* ranged from 56 to 88 between different accessions. The genetic distance of 88 was exhibited among many *G. indica* accessions. The accession GI_MDG_4 showed significant genetic distance of 88 with most of the other accessions, while the least genetic distance of 56 was exhibited between GI_KOK_1 and GI_KAR_15 among all the accessions. The allele frequencies across loci were assessed with Graph over Loci for Codominant Data, where two markers- ITBTGI8 and ITBTGI10 showed higher allelic frequencies followed by markers ITBTGI2 and ITBTGI1. ITBTGI4, ITBTGI18 and ITBTGI20 recorded low allelic frequency among the 22 markers across loci. The Graph over Loci for Codominant Data for *G. indica* accessions is presented in Figur 1. Similar results were reported by Roy *et al.* (2015) in *G. indica*

Principal Coordinate Analysis (PCA) carried out to understand the relationships between the sampled accessions of *G. indica* based on genetic distance and is represented in Figure 2. Both upper and lower quadrants on the right represented *G. indica* accessions sampled from Uttara Kannada and Dakshina Kannada whereas, the left upper quadrant consisted of one accession sampled from Udupi along with accession collected from Uttara Kannada and Dakshina Kannada. The lower left quadrant represented the one accession sampled from Chikmagalur along with accession from Uttara Kannada and Dakshina Kannada.

The PCA obtained in the present study separated the accessions as well as individuals based on their geographical distribution which is possibly due to high degree of diversity between different populations than within a particular population. Similar results were obtained for ISSR marker profiling of different *Garcinia* species the from *Western Ghats* and Northeastern Himalayas, wherein, *Garcinia* species were separated into different quadrants based on the geographical distribution (Parthasarathy *et al.,* 2013). The genetic diversity and population structure of a species are well understood by analyzing the differences in allelic frequencies and phylogenetic relationship of individuals in a population and among different populations across the different geographical regions. Phylogenetic analysis clearly distinguishes a genetically distinct individual or a population from the rest (Sivu *et al.,* 2017) and Chinavat and Subadrabuddhe (2004). In *Garcinia* species, analysis of the phylogeny of populations is an important step in understanding the relationships among the populations and their evolution.

The Neighbor--Joining (NJ) phylogenetic tree constructed across 19 accessions of *G. indica* and represented in Figure 3. In the present study, two major clusters existed *viz.,* Cluster 1 and Cluster 2. The cluster 1 was further sub-divided into two sub-clusters (C1a and C1b). Subcluster C1a consisted of accessions sampled from Uttara Kannada and Dakshina Kannada regions, whereas the sub-cluster C1b consisted of accessions sampled from Udupi, Uttara Kannada and Dakshina Kannada. On the other hand, cluster C2 consisted of accessions from Chikmagalur along with accessions from Uttara Kannada and Dakshina Kannada. This type of genetically admixured clusters obtained by Randomly Amplified Fingerprinting (RAF) of mangosteen (*Garcinia mangostana*) ecotypes collected from different regions of Australia (Sando *et al.,* 2014).

The results obtained for DNA fingerprinting analysis indicated that microsatellite ITBTGI1 recorded maximum allelic size of 1000 bp for the accession G.I-KAN 3, whereas, the microsatellite ITBTGI12 exhibited least allelic size of 107 for the accession G.I-KAN20. Three different microsatellites *viz.* ITBTGI4, ITBTGI16 and ITBTGI20 recorded different allelic sizes of 218, 184 and 220 bp respectively for the accession G.I-SIR 5. The allelic sizes of the PCR amplified products using these markers for all accessions are-presented in Table 4.3.2. A total of 25 SSRs were used to assess fingerprinting among the 19 genotypes. Out of 25 SSRs, 7 showed clear polymorphic amplification profiles. Results from the present study indicated the presence of a vast genetic diversity among the analyzed *G. indica* and *G. caombogia* accessions. Similar results were also reported by Begum *et al*., (2012) indicating the occurrence of an intense gene flow between these two varieties. DNA fingerprints obtained by hybridization of mini-satellite probes with genomic DNA was first reported by Adato *et al*. (1995). Subsequently, DNA fingerprints were developed for 59 Florida mango cultivars using capillary electrophoresis by Schnell *et al*. (2005), but such fingerprints were not based on genotype-specific allele sizes. Viruel *et al*. (2005) distinguished 28 mango genotypes with just three SSR markers. Cultivar-specific alleles of six varieties were obtained by Pandit *et al*. (2007) by using a combination of eight ISSR primers based on 2 per cent agarose gel electrophoresis.

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