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Comparative effectiveness of inter-simple sequence repeat and randomly amplified polymorphic DNA markers to study genetic diversity of Indian *Garcinia*

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A study to compare the effectiveness of inter-simple sequence repeats (ISSR) and randomly amplified polymorphic DNA (RAPD) profiling was carried out with a total of 65 DNA samples using 12 species of Indian *Garcinia*. ISSR and RAPD profiling were performed with 19 and 12 primers, respectively. ISSR markers generated a total number of 156 bands with 92 polymorphic bands, while RAPD markers produced a total of 134 bands with 80 polymorphic bands. Percentage of polymorphic loci in RAPD profiling was 60.4% while in ISSR profiling, it was 59.3%. Heterogeneity index was similar for the markers, 0.86 for ISSR and 0.89 for RAPD, indicating that both the marker systems are effective in determining polymorphism in *Garcinia*. ISSR markers showed clear distinction among the species whereas RAPD markers showed segregation based on geographical location as well as species based.

Key words: *Garcinia*, genetic diversity, inter-simple sequence repeats, randomly amplified polymorphic DNA, principal component analysis.

INTRODUCTION

Garcinia is a large genus with 240 species of evergreen trees and shrubs. About 35 species are reported in India, among which seven are endemic to Western Ghats, 5 in north-east Himalayas and six in Andaman Islands (Peter and Abraham, 2007). *Garcinia* is one among the few genera in angiosperms that shows a very high degree of diversity (Osman and Rahman, 2006). It is one of the potential under exploited multipurpose crops and recently gained a lot of attention as a popular means of weight loss because of the presence of (-) hydroxycitric acid in the fruit rind and leaves. The Plant List (2010) reports that 25.5% of the species names of *Garcinia* are synonyms, 3.4% names are unplaced and 4.4% names are unassessed. The differences that distinguish one plant from another are encoded in the plant's genetic material, the DNA. So the difference among the species and within the species can be studied using various molecular markers. ISSR and RAPD are the two commonly used PCR based techniques to study genetic variation among the species. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Pradeep et al., 2002). This technique is useful for phylogeographic analyses or

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Abbreviations: BSI, Botanical Survey of India; **ISSR,** inter-simple sequence repeats; **RAPD,** randomly amplified polymorphic DNA; **PMB,** percentage of monomorphic bands; **PPB,** percentage of polymorphic bands; **PIC,** polymorphism information content; **PCoA,** principle coordinate analysis.

delimiting species.The RAPD technique has received a great deal of attention from population geneticists because of its simplicity and rapidity in revealing DNA-level genetic variation. Congiu et al. (2000) employed RAPD markers for individualization of strawberry. RAPD and ISSR markers were utilized for comparative analysis of genetic diversity in blackgram genotypes (Souframanien and Gopalakrishna, 2004). A work by Seyit et al. (2010) showed a comparison of RAPD and ISSR markers for the genetic characterization of seized *Cannabis sativa* L. The study shows the effectiveness of these molecular markers through various statistical analyses. Similarly, comparison of ISSR and RAPD markers was performed to analyze their efficacy in determining genetic diversity of pistachio (Ayda et al., 2010), apricots (Meetul et al., 2009) and barley (Ferdaous et al., 2012).

The objective of this study was to compare the effectiveness of ISSR, RAPD and ISSR-RAPD combined system to find out the molecular diversity of *Garcinia* species of various geographical regions in India.

MATERIALS AND METHODS

Plant materials and DNA extraction

Leaf samples from a total of 65 plant samples were collected from two distinct geographical locations, namely, North Eastern Himalayan foot hills (namely Assam, Nagaland and Meghalaya) and from Western Ghats (namely Kerala, Tamil Nadu, Karnataka and Maharashtra). The species are *G. indica* Chois., *G. gummi-gutta* Robs., *G. cowa* Roxb., *G. kydia* Roxb., *G. lanceaefolia* Roxb., *G. xanthochymus* Roxb., *G. pedunculata* Roxb., *G. morella* Desrouss., *G. nervosa* Miq., *G. dulcis* (Roxb.) Kurz., *G. cornea* Linn. and *G. spicata* (Wight & Arn.) Hook. Information relevant to samples are given in Table 1. Genomic DNA was isolated from the leaves of the plants by modified Doyle and Doyle (1990) method using 4% CTAB buffer containing 1% PVP. Purified DNA was quantified in agarose gel electrophoresis and Eppendorf BioPhotometer and suitably diluted.

Molecular marker profiling

Thirty (30) RAPD primers were used for initial screening, out of which 12 primers were found to be suitable for amplification. The sequences of the primers used are given in Table 2. All the primers were decamers. For ISSR profiling, 55 UBC primers available in the institute were first screened *in-silco*. The selected 19 primers were used for wet-lab studies. The selected primers were 18 and 20-*mers* of various GA and CA repeats. The sequences of the primers used are given in Table 3. The concentration of reaction mixture components and the PCR conditions were standardized by trials. Reactions were carried out in a total volume of 25 µl containing 50 ng DNA, 1 µl each of primer (20 pM) and dNTP (10 mM) solution. 2.5 µl 10xTaq buffer (without $MgCl_2$) and 0.2 µl of 5U Taq polymerase from Merck (Genei Pvt. Ltd., Bangalore) was used for the reaction. For RAPD, 1.5 µl of 25 mM $MgCl_2$ was used while 1 µl for ISSR. The rest of the volume was made up using nuclease free sterile water. For ISSR profiling, amplification was performed under the following conditions: 5 min at 94°C for 1 cycle, followed by 30 s at 94°C, 1 min at annealing temperature (depending on primer) and 1 min at 72°C for 35 cycles. Final extension was done at 72°C for 10 min. For RAPD profiling, denaturation step was performed for 1 min and final extension for 15 min. The rest steps remained unchanged.

PCR products were resolved by agarose gel (1.5% in 1xTAE buffer, containing 1% ethidium bromide) electrophoresis and then documented using Kodak gel documentation-scoring system.

Data analysis

Various molecular profile characteristics such as total number of bands, percentage of monomorphic and polymorphic bands, polymorphic information content (PIC) and Mantel correlation (r) were tabulated separately for RAPD and ISSR profiling. Rectangular binary data matrices were created for ISSR and RAPD profiling, with 1 for the presence of band and 0 for absence, which were used for further statistical studies. Binary matrices of ISSR and RAPD were combined (ISSR-RAPD) to facilitate an analysis of combined ISSR and RAPD markers together as well. Pair-wise similarity matrices were generated using SIMQUAL procedure of NTSYS statistical package. Principal coordinate analyses (PCA) and (PCI) of data were also performed using NTSYS. Cluster analyses for the matrices were performed by means of SAHN procedure via UPGMA to develop dendrograms. Mantel test was performed in order to compare ISSR versus RAPD, ISSR versus ISSR-RAPD and RAPD versus ISSR-RAPD.

RESULTS AND DISCUSSION

In this study, a total number of 19 UBC ISSR primers and 12 UBC RAPD primers were used for the detection of polymorphism. Figures 1 and 2 represent the good polymorphism with UBC -ISSR 810 and UBC-RAPD-PO5. Various molecular profiling characters, namely, percentages of monomorphic bands (PMB) and polymorphic bands (PPB) for each primer and their polymorphism information content (PIC) for ISSR profiling is given in Table 2 and that for RAPD analysis is given in Table 3. The ISSR and RAPD markers used allowed reproducible and informative polymorphisms (Figures 3, 5 and 7). Seyit et al. (2010) also recorded the same type of work with marijuana (*Cannabis sativa L*). Figures 1 and 2 represent the banding pattern by *Garcinia* species indicating considerable level of polymorphism. In ISSR profiling, largest number of monomorphic bands were produced by primers 810 and 815 (3 bands), whereas primers 816 and 848a produced only polymorphic bands. Primers 857a and 857b amplified highest numbers of polymorphic bands with an average of 5.3 and 4.8 bands per sample, respectively. The total number of bands was highest in 857a, producing an average of 7.2 bands per sample. ISSR primers generated a total of 157 with an average of 8 bands per primer, out of which 63 bands were monomorphic. Hakki et al. (2007) also reported effective segregation of species with ISSR. Kojoma et al. (2002) also used ISSR for studying DNA finger printing in *C. sativa*.

RAPD primers generated a total of 134 bands, average of 11.2 per primer, out of which 53 bands were monomorphic. Primers AO12 and AB16 produced largest number of monomorphic bands (two bands). AB01 primer

Table 1. *Garcinia* accessions and corresponding locations.

Sample number	Species	Location	Ecosystem
1, 2, 3, 4, 5, 6, 7, 8 and 9	*G. xanthochymus*	Assam	N. E. Himalayan foothills
38, 39 and 40		Meghalaya	
10, 11, 12 and 13	*G. kydia*	Assam	
30, 31, 32, 33, 34 and 35		Meghalaya	
41, 42 and 43		Nagaland	
29	*G. cowa*	Assam	
36 and 37		Meghalaya	
14, 15, 16 and 17	*G. pedunculata*	Assam	
18, 19, 20, 21, 22 and 23	*G. lanceaefolia*	Assam	
24	*G. nervosa*	Assam	
25	*G. spicata*	Assam	
26	*G. morella*	Assam	
27	*G. dulcis*	Assam	
28	*G. cornea*	Assam	
44, 53, 54, 55 and 56	*G. gummi-gutta*	Karnataka	Western Ghats
47, 48, 49, 50, 51 and 52		Kerala	
45 and 46		Tamil Nadu	
60, 64 and 65	*G. indica*	Karnataka	
61, 62, 63		Kerala	
57, 58 and 59	*G. xanthochymus*	Kerala	

Table 2. ISSR primers and details of the profiling.

Code	Sequence	TNB	PMB	PPB	Band range (bp)	PIC_i
868	(GAA)6	8	56.56	43.44	394.7 - 1384	0.94
816	(CA)8T	7	0.00	100.00	238 - 875	0.90
810	(GA)8T	13	69.31	30.69	263.2 - 933.3	0.95
812	(GA)8A	7	31.11	68.89	217.4 - 730.8	0.74
815	(CT)8G	10	73.43	26.57	522.7 - 2031	0.95
835a	(AG)8CC	5	42.86	57.14	485 - 2000	0.93
835b	(AG)8TC	11	49.62	50.38	272 - 1366.7	0.90
841a	(GA)8CC	6	51.11	48.89	280.6 - 2000.5	0.87
841b	(GA)8TC	16	32.75	67.25	275.4 - 2100	0.92
848a	(CA)8AG	7	0.00	100.00	250 - 890.5	0.80
848b	(CA)8GG	5	24.18	75.82	250 - 933.2	0.90
860a	(TG)7AA	5	59.57	40.43	522.7 - 2200.2	0.95
860b	(TG)8GA	6	42.42	57.58	500.3 - 2100	0.98
861	(ACC)6	8	64.40	35.60	235 - 750.5	0.86
852a	(TC)8AA	8	46.46	53.54	250.8 - 730.6	0.93
857a	(AC)8CG	13	26.82	73.18	510.4 - 2100	0.75
809	(AG)8G	5	50.00	50.00	500 - 2000.2	0.79
840b	(GA)8TT	5	23.75	76.25	395 - 1530.6	0.90
857b	(AC)8TG	12	28.33	71.67	475.5 - 2200.3	0.75
Average		8.2	40.67	59.33		0.86

Table 3. RAPD primers and details of the profiling.

Code	Sequence	TNB	PMB	PPB	Band range (bp)	PIC_i
AB 01	CCGTCGGTAG	18	32.31	67.35	173.2 - 1500	0.90
AA 01	AGACGGCTCC	13	42.61	57.39	160.1 - 3214.2	0.91
AP 20	CCCGGATACA	8	46.54	53.46	200 - 1750.3	0.90
AV 03	TTTCGGGGAG	7	52.30	47.70	110 - 1277.7	0.91
AO 12	TCCCGGTCTC	12	58.78	41.22	525.4 - 3200	0.91
BB 18	CAACCGGTCT	8	31.41	68.59	200.5 - 1800	0.84
AB 16	CCCGGATGGT	12	51.24	48.76	295.6 - 2000	0.86
AB 11	GTGCGCAATG	14	25.33	74.67	300 - 1914.2	0.93
AA 11	ACCCGACCTG	10	33.82	66.18	388.4 - 2000	0.93
W 15	ACACCGGAAC	14	36.03	63.97	200 - 1777.3	0.90
PO 5	CCCCGGTAAC	9	29.33	70.67	110 - 1714.3	0.92
AF 11	AAGACCGGGA	9	34.90	65.10	200.5 - 2285.5	0.79
Average		11.2	39.55	60.42		0.89

TNB - Total number of bands, PMB - percentage of monomorphic bands, PPB- percentage of polymorphic bands, PIC_i - polymorphic information content.

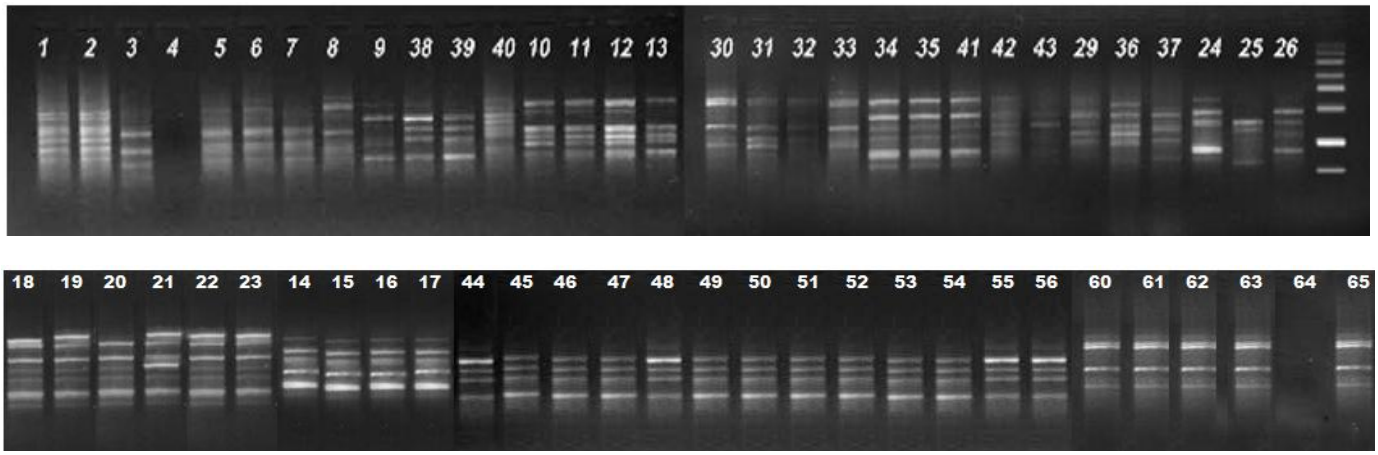

Figure 1. Gel picture for ISSR profiling using primer 810.

amplified highest number of polymorphic bands with average of 3.2 bands per sample. This primer also produced highest total number of bands (4.3 bands per sample). Percentage of polymorphic loci in RAPD detected was 60.42%, while in ISSR profiling it was 59.33%. Average percentages of monomorphic bands and polymorphic bands for ISSR profiling were 40.6 and 59.3 respectively and for RAPD they were 39.5 and 60.4, respectively. Though there were some variation in the total number of bands, PMB and PPB percentages were almost same. The dendrograms constructed using RAPD and ISSR marker profiling did not show considerable degree of variation with each other as revealed by Mantel correlation (r = 0.558). The similarity coefficient ranged 0.66 to 1.00 with ISSR, 0.60 to 0.96 with RAPD and 0.68 to 0.98 for the combined RAPD-ISSR dendrograms. Seyit et al. (2010) reported a weak correlation of (r = 0.03) in ISSR and combined ISSR-RAPD matrix for marijuana of Turkey. A total of seven clusters were observed in dendrogram (Figure 3) for ISSR profiling. *G. xanthochymus* from both ecosystems formed cluster 1. The other clusters were of *G. pedunculata*, *G. lanceaefolia*, *G. kydia*, *G. cowa*, *G. gummi-gutta* and *G. indica*. The clusters of *G. gummi-gutta* and *G. indica* were distinct from the other clusters which contain the species of Himalayan origin. Species with single accessions such as *G. nervosa*, *G. spicata*, *G. morella*, *G. dulcis* and *G. cornea* (24 to 28) which were collected from BSI Sibpur, germplasm were separated out as cluster 3 in Figure 3. Similarly, in the PCoA scattered diagram (Figure 4), the Himalayan species were clustered nearby while Western Ghats species had separated out. Even though *G. xanthochymus*,

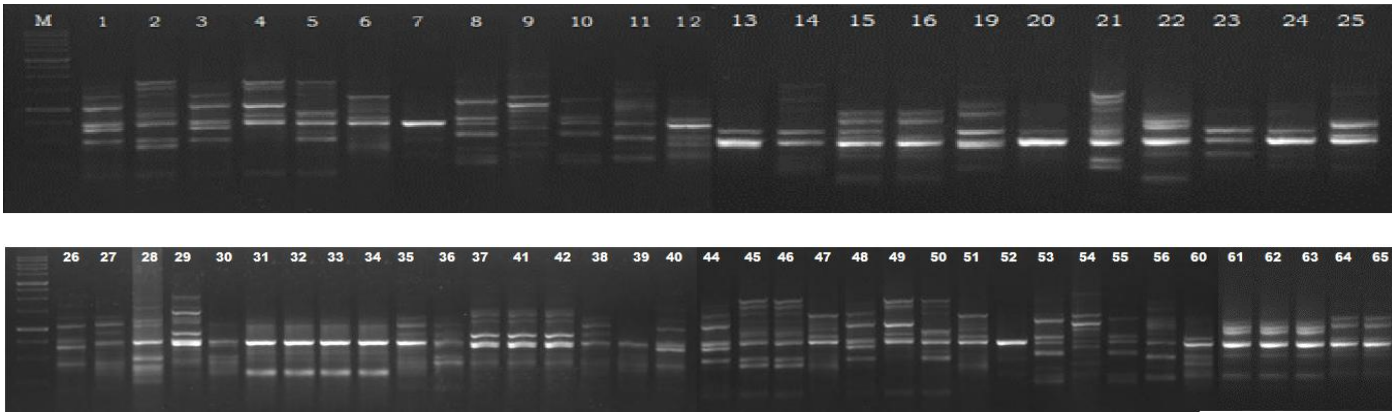

Figure 2. Gel picture for RAPD profiling using primer PO 5.

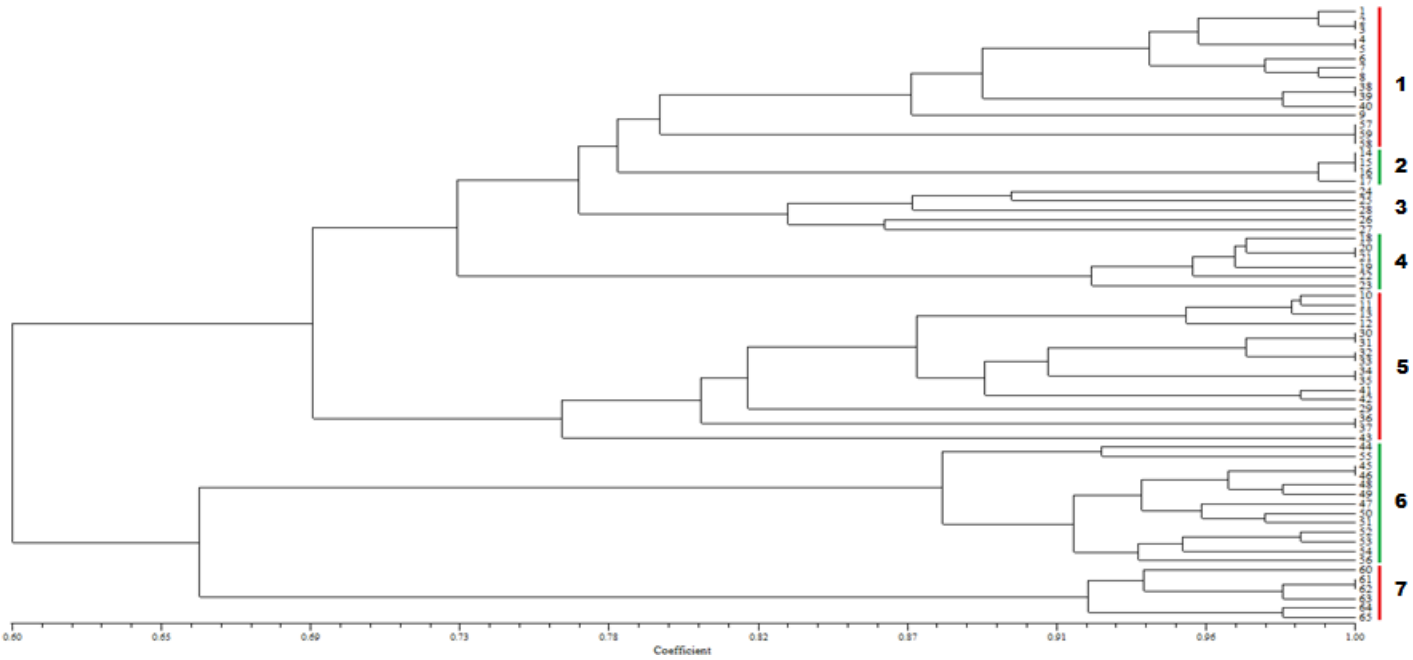

Figure 3. Dendrogram for ISSR profiling.
Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 -(24, 25, 26, 27and 28 single accession collected from BSI, Kolkata), 4 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 5 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 6 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56) 7 - *G. indica* (60, 61, 62, 63, 64 and 65).

the species collected from Western Ghats was clustered together with the Himalayan species, it showed a slight segregation.

The dendrogram for RAPD profiling also produced 6 clusters (Figure 5), which showed species distinction. The PCoA analysis showed only 3 clusters. In the PCoA analysis, *G. xanthochymus*, of both the region came together while *G. gummi-gutta/G. indica* formed a combined cluster (Figure 6). The rest of the species separated out. Here the clustering pattern represented their origin and natural habitat. In this case distinctions between species were not much clear. The species with single accession (*G. nervosa*, *G. spicata*, *G. morella*, *G. dulcis* and *G. cornea*) were randomly dispersed in the clusters with Himalayan species. Seyit et al. (2010) also got very clear segregation in the PCA analysis of the two (ISSR & RAPD) systems, in their studies on *Cannabis*. In case of the combined cluster of ISSR and RAPD profiling, the pattern of dendrogram (Figure 7) was found similar to that of ISSR profiling. The Western Ghat and Himalayan

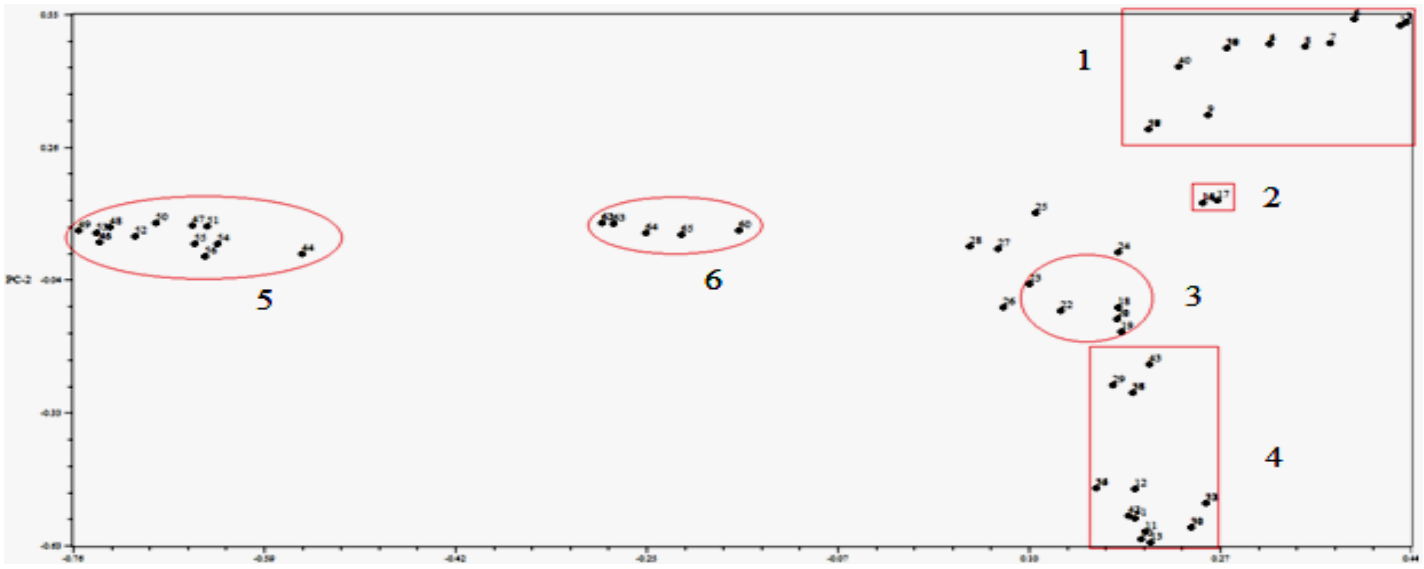

Figure 4. Principle coordinate analysis for ISSR profiling. 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4 - *G. kydia*, - *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 5 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 6 - *G. indica* (60, 61, 62, 63, 64 and 65).

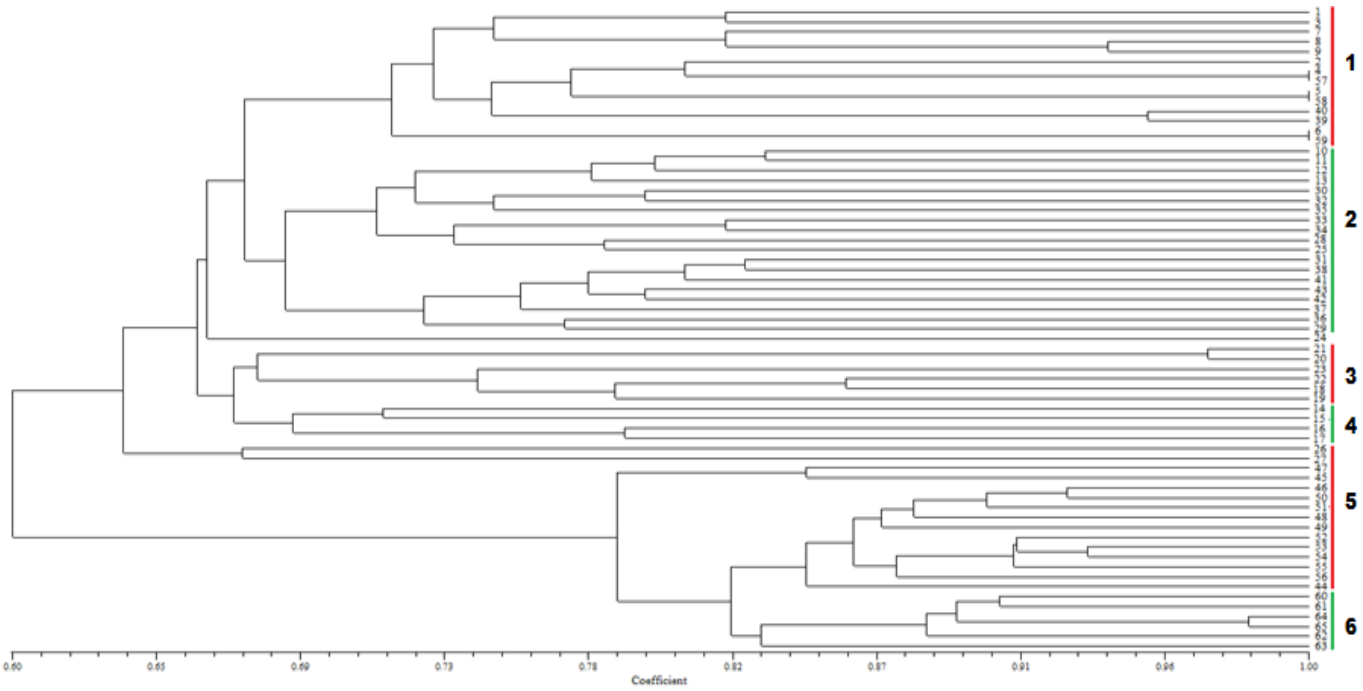

Figure 5. Dendrogram for RAPD profiling. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 3- *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4- *G. pedunculata* (14, 15, 16 and 17), 5- *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 6- *G. indica* (60, 61, 62, 63, 64 and 65).

species were clustered with clear distinction. In the case of PCoA analysis (Figure 8), *G. gummi-gutta* and *G. indica* formed one cluster which is distinctly separated from the clusters formed by other species.

Conclusion

The study indicates that in *Garcinia*, both the marker systems were equally effective. Both ISSR and RAPD

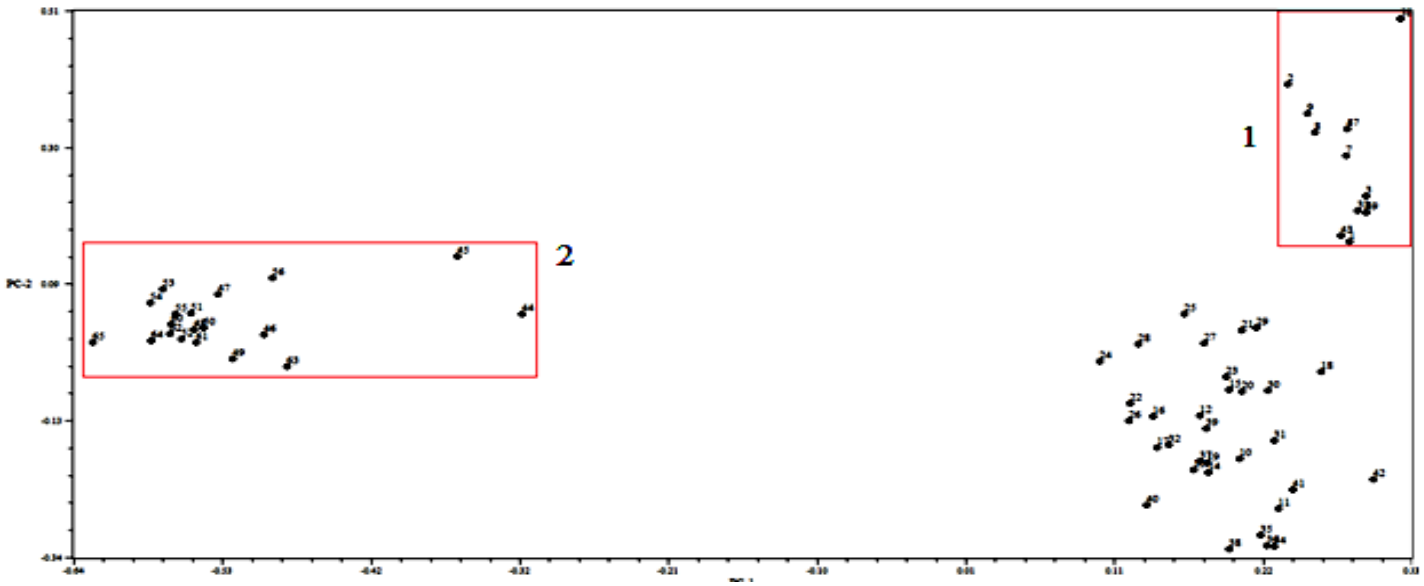

Figure 6. Principle coordinate analysis for RAPD profiling. Cluster 1- *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. gummi-gutta* and *G. indica* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 60, 61, 62, 63, 64 and 65).

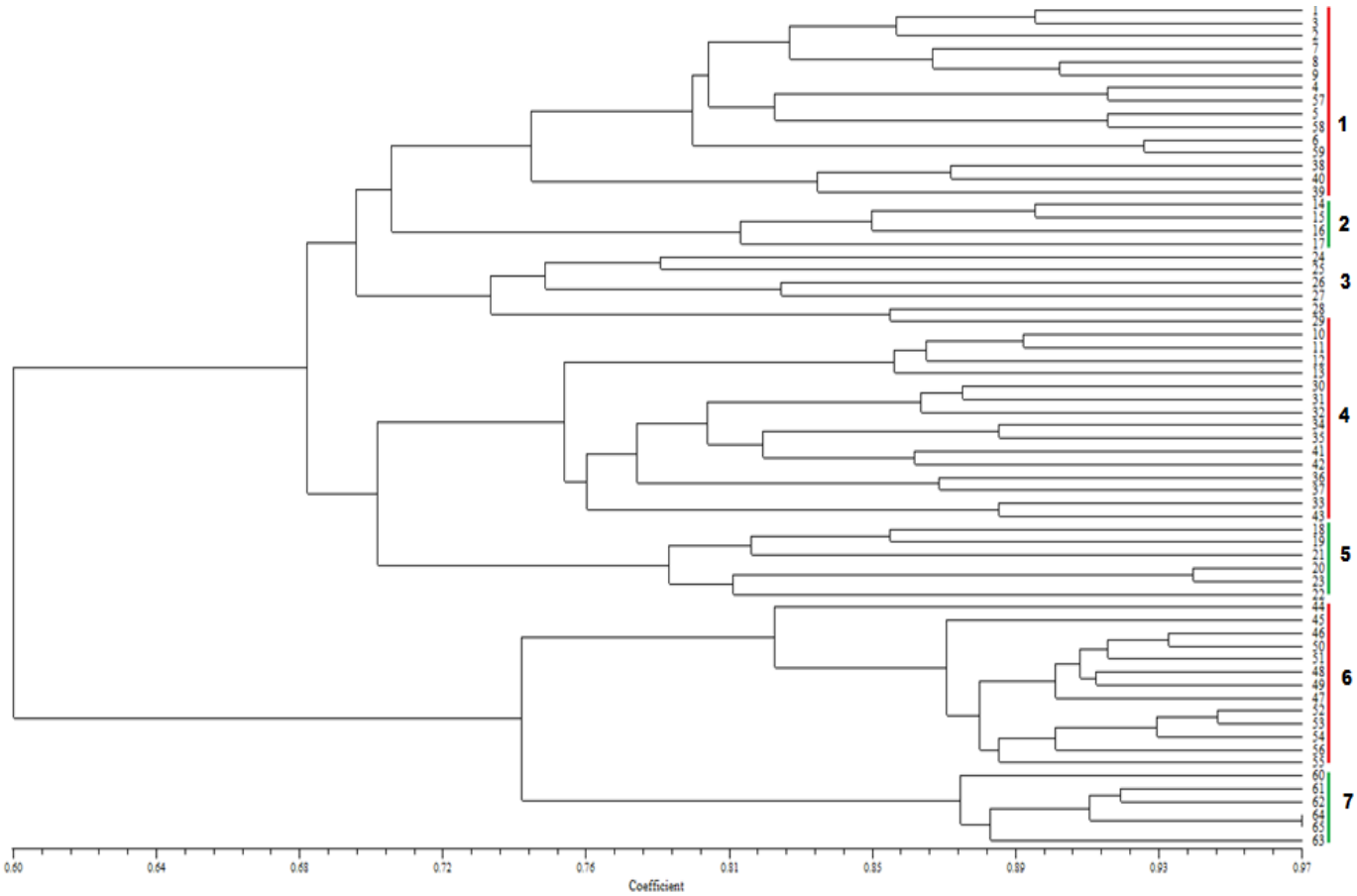

Figure 7. Dendrogram for RAPD-ISSR combined data matrix. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16, and 17), 3 - (24, 25, 26, 27, and 28), 4 - *G. lanceaefolia* (18, 19, 20, 21, 22, and 23), 5 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42, and 43), 6 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 7 - *G. indica* (60, 61, 62, 63, 64 and 65).

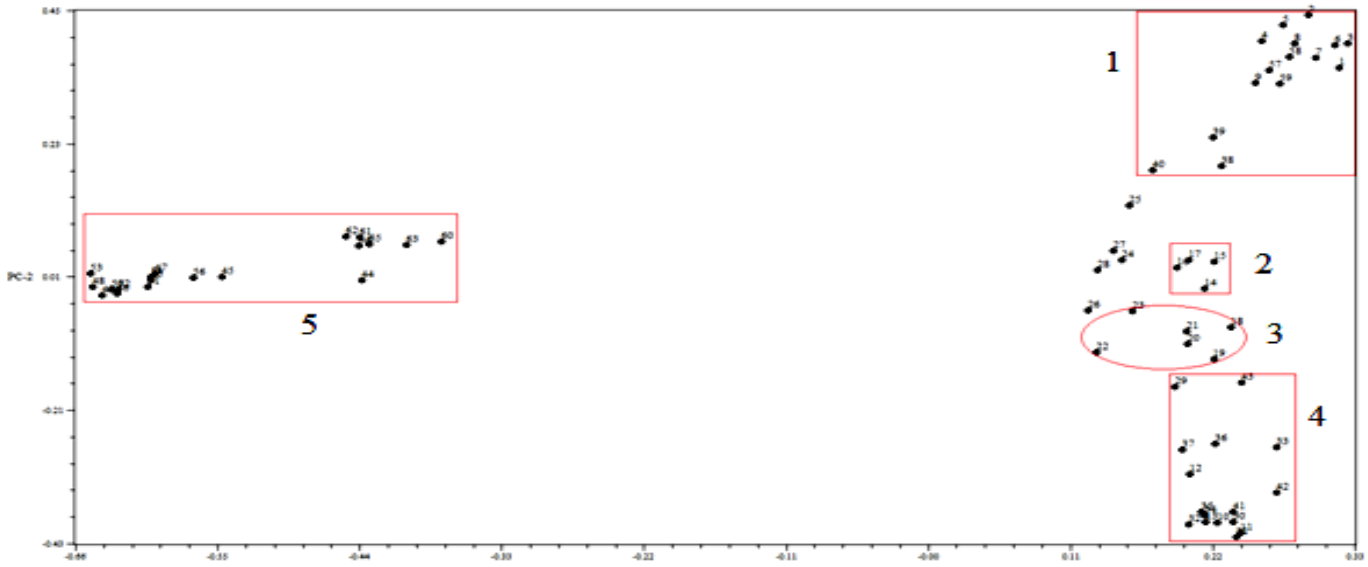

Figure 8. Principle coordinate analysis for RAPD-ISSR combined data. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42, and 43), 5 - *G. gummi-gutta*, *G. indica* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 60, 61, 62, 63, 64 and 65).

markers produced polymorphism and proved helpful for diversity studies. PCR based marker systems are best to analyze genetic relationship of crops (Milbourn et al., 1997; Patzak, 2001). It is interesting to note that average band for ISSR was 8.2, while that of RAPD was 11.2. The highest BP value in case of ISSR was 2200 kb, and 9 primers showed bands more than 2000 kb. In case of RAPD, the highest bp value was 3214 kb and in all the samples highest value is more than 1500 kb. In case of lowest bp value, RAPD showed 110 kb and ISSR was 235 kb. In comparison to ISSR, RAPD gave larger ranges of bands. ISSR primers could give a distinct segregation based on the species of the sample, whereas RAPD primers produced clustering based on geographical location of the samples (Jagadish et al., 1996). PIC values for both the marker systems are very close, indicating the suitability of both the marker types in molecular studies.

This study indicates that combined information of ISSR and RAPD markers could be a better tool in studying molecular diversity of *Garcinia*, especially as it does not have adequate genomic information to design specific markers (Seyit et al., 2010). It can be concluded from the present study that ISSR markers could be used to identify the unknown species in *Garcinia*, whereas RAPD markers can be used to determine the variation based on geographical or morphological features within the same species.

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