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Genetic Diversity among Jamun (*Syzygium cumini* L. Skeels) Accessions of DNA markers

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Abstract

Jamun (*Syzygium cumini* L. Skeels) is one of the most important wild fruit trees having great medicinal and economic value. Characterization of Jamun collected from Andaman and Nicobar Islands, India was undertaken through morpho – biochemical and using DNA marker technology. A set of 10 ISSR and 15 RAPD primers were taken for DNA fingerprinting, wherein 5 ISSR and 7 RAPD primers produced 38 and 52 amplicons out of which 24 and 31 amplicons were polymorphic having 63.15 and 59.61% polymorphism, respectively. The highest polymorphism was revealed by IS12 (78%) in ISSR and OPF 1 (75%) in RAPD primers. ISSR analysis revealed precise information on polymorphism as compared to RAPD primers. The average polymorphism of 22 accessions using 12 primers was 61.11 %. The characterization of Jamun accessions would help in utilizing them for fruit quality and yield besides conservation efforts.

Key words: *Syzygium cuminii*, Jamun, Molecular markers, biodiversity, Andaman and Nicobar Islands

Introduction

Jamun (*Syzygium cumini* Skeels) is a known underutilized minor fruit species native to India. It belongs to the family Myrtaceae and shows wide variability for number of traits with versatile adaptability to a wide range of ecological regions in tropical and subtropical zones of is an important minor fruit of Indian origin, commonly known as Black plum, found growing widely in different agro-climatic conditions. (Kirtikar and Basu, 2006). Besides it is grown throughout South East Asia, Malaysia, Myanmar and Sri Lanka. It is reported to be used in cure of diabetes due to its phyto-constituents comprising alkaloids jambosine, glycoside, antimellin, phenolic -gallic acid and ellagic acid. The fruits are a good source of iron and are used as an effective medicine against diabetes, heart and liver trouble (Shrivastava and Kumar, 2009). The enormous variability has been reported in *S. cumini* due to inherent heterozygosity as cultivars are mainly propagated through seedling selections. Andaman and Nicobar Islands harbour a large number of under - utilized fruit crops, including ten *Syzygium* species, among which *S. cumini* has high variability and distributed throughout the islands. Assessment of genetic variability is considered vital tool for formulating conservation strategies of this underutilized fruits. Traditional methods for assessment of genetic variation among accessions based on the morphological characteristics are often biased since most of these characteristics are influenced by environmental factors and plant developmental stage. Furthermore, phenotypically indistinguishable accessions or species may or may not be genotypically similar with availability of limited number of morphological descriptors. During the last decade, classical methods have been complemented with molecular techniques for evaluating genetic variation as they are more reliable tool for distinguishing the cultivars.

RAPD and Inter-Simple Sequence Repeats (ISSR) markers have been extensively used for the species/cultivar identification in a wide range of plants (Mariniello *et al*., 2002) as they does not require prior knowledge about the

genome of plants. They are simple to use, efficient and provide a quick method for identification of genotypes at any developmental stage (Conner *et al*., 2001). An ISSR marker amplify inter-microsatellite sequences at multiple loci throughout the genome (Li et al., 2005) and permit the detection of polymorphism in microsatellites and inter microsatellite loci without previous knowledge of DNA sequences. Information on the genetic diversity within and between closely related crop varieties is essential for rational use of genetic resources (Kalyan and Rambabu, 2006). Due to lesser availability of superior recommended varieties of jamun, the growers have been planting trees of either seedling origin or grafted plants of unknown yield potential and fruit quality, which resulted in wide variation for flowering, fruit yield and quality. Hence, the present study was conducted to elucidate the variation for morphological as well as physicochemical characteristics of jamun trees through conventional and molecular approaches using DNA based markers *viz*., ISSR, RAPD with an objective to identify superior jamun clones in Andaman and Nicobar Islands.

Material and methods

Survey was conducted in Andaman and Nicobar Islands, India among the jamun trees both in cultivated state as well as in natural forest stand as per method suggested by Gupta and Rai (1996) during 2015 – 2016 and 22 high yielding jamun accessions were identified(Table 1). The extent of variation in fruit physio-chemical traits from different locations was assessed. Ten fruits from selected trees were randomly taken for measuring physical attributes like fruit length, fruit diameter, length diameter ratio, fruit weight, pulp weight, seed weight and pulp to seed ratio following standard procedures. Total soluble solids (%) were estimated with the help of hand refrectometer. Titrable acidity was estimated by titrating 10 ml juice against 0.1 N NaOH using phenolphthalein as indicator (AOAC, 1960). Reducing and total sugars were determined by volumetric method as suggested by Lane and Eynon (1923). Ascorbic acid content of fruits was determined using standardized 2, 6-dichlorophenol indophenol dye and expressed as mg per 100 g of pulp. The data were analyzed as per the method suggested by Gomez and Gomez (1984) using randomized block design.

Table. 1: Geographical origin of various accessions of *Syzygium cumini* in Andaman & Nicobar Islands.

Accession No.	District	Altitude (MSL)	Latitude	Longitude
1	South Andaman	34	N 11°65′61.4″	E 92° 73′ 62.4″
2	South Andaman	28	N 11° 60′19.1″	E 92° 58′ 43.1″
3	South Andaman	26	N 11° 36′95.9″	E 92° 44′ 91.0″
4	South Andaman	36	N 11° 53′58.4″	E 92° 72′ 81.4″
5	South Andaman	31	N 11° 59′86.3″	E 92° 68′ 10.7″
6	South Andaman	25	N 11° 64′95.3″	E 92° 73′ 15.5″
7	South Andaman	26	N 11° 65′12.7″	E 92° 73′44.9″
8	South Andaman	30	N 11° 67′29.5″	E 92° 76′24.9″
9	South Andaman	69	N 12° 56′80.2″	E 92° 81′ 29.1″
10	South Andaman	74	N 11° 59′83.5″	E 92° 71′43.5″
11	South Andaman	56	N 11° 65′97.3″	E 92° 74′ 73.7″
12	South Andaman	56	N 11° 66′21.7″	E 92° 75′ 60.5″
13	South Andaman	43	N 11° 66′66.3″	E 92° 74′ 89.0″
14	South Andaman	3	N 11° 66′17.3″	E 92° 47′ 62.4″
15	South Andaman	25	N 11° 65′47.3″	E 92° 73′ 72.7″
16	North & Middle Andaman	8	N 12° 91′56.6″	E 92° 89′ 43.1″
17	North & Middle Andaman	68	N 12° 39′89.5″	E 92° 93′ 76.1″

18	North & Middle Andaman	47	N 12° 50′51.8″	E 92° 91′50.5”
19	North & Middle Andaman	18	N13° 52.6′18.4″	E 92° 98′ 49.6″
20	Nicobar	76	N 7° 07 ′56.5″	E 93° 90′ 84.9″
21	Nicobar	80	N 7° 03′89.8″	E 93° 87′ 23.7″
22	Nicobar	19	N 9° 21′ 71.2″	E 92° 72′ 62.4″

The fresh disease free healthy seeds of all the 22 accessions (Acc.) were sown in to the poly bags and the seedlings used for DNA extraction. The total genomic DNA was extracted from disease free fresh young leaves by CTAB method (Doyle and Doyle, 1987) with slight modification. Purity of DNA was checked by UV spectrophotometer and ran on 1% agarose gel. The quantification of DNA in RNA free sample was done using UV spectrophotometer. PCR reaction was performed in final volume of 20 µl containing 10x assay buffer 2.5 mM dNTPs, 0.5 unit of *Taq* DNA polymerase all from Bangalore Genei@, 10 pmols/reaction ISSR/RAPD primer and 100 ng of template DNA. Analysis of ISSR markers was done with 10 primers (Clonitec) and that for RAPD markers was done with 15 primers obtained from OPERON TECHNOLOGIES Inc. Alameda Calif.

The PCR was performed by initial denaturation at 94°C for 5 min. followed by 45 cycle of denaturation at 94°C for one min., annealing at 37°C for one minutes, and extension at 72°C for two minutes and final elongation of at 72°C for 7 min. For ISSR the annealing temperature were taken as recommended by company. The PCR products were resolved on 1% Agarose gel (Bangalore Genei@) prepared in 1 × TAE buffer containing 0.5 µg/ml of the ethidium bromide at 100 V for 2.5 h. All the genotypes were scored for the presence and absence of the ISSR and RAPD bands and the data were entered into a binary matrix as discrete variables. The value of 1 was assigned for presence and 0 for absence of character and this data matrix was subjected to further analysis. The 0/1 matrix was used to calculate similarity using Jaccard's coefficient. The resultant similarity matrix was employed to construct dendrogram using SAHN based UPGMA to infer genetic relationship (Rohlf, 1998; Sneath *et al*., 1973).

Results and Discussion

The data pertaining to physical and chemical quality attributes of jamun fruits showed significant differences and a high degree of variability for all the characters studied. The fruit weight varied from 1.89 g in Acc.-7 to 18.60 g in Acc.-3 thus, higher fruit weight is a preferred character in jamun. Higher length: diameter ratio indicated the cylindrical shape, while lower ratio suggested the oblong and round shape of the fruits. Maximum length and diameter ratio (1.85) was recorded in Acc.-17, which exhibited cylindrical fruits. Variation in jamun genotypes with respect to above characters was earlier reported from Goa (Devi *et al*., 2002) and West Bengal (Kundu *et al*., 2001). Pulp weight, seed weight and pulp:seed ratio also varied significantly (Table 2). The maximum pulp weight was recorded in acc-3 and lowest pulp weight was recorded in Acc- 7. In all the 22 accessions no relation was observed with respect to pulp content, fruit weight and length to diameter ratio, which may be attributed to the variable weight of the seed. The maximum seed weight was recorded in Acc.-3 and minimum in Acc. - 15. Lower seed weight is a preferred character for table purpose jamun. This variation in fruit weight and seed weight might be due to genetic makeup of the accessions as well as the prevailing microclimates.The above observations revealed that while selecting a superior jamun genotype, pulp weight should be given more importance rather than the fruit weight. The pulp to seed ratio in various accessions ranged from 1.39 in Acc. - 7 to 6.78 in Acc. - 4 and showed wide range of variability. Similar results were also reported from Goa (Devi *et al*., 2002).

Table. 2. Fruit attributes of jamun accessions

Accession No.	Fruit length (mm)	Fruit diameter (mm)	Length : diameter ratio	Fruit weight (g)	Pulp weight (g)	Seed weight (g)	Pulp seed ratio
1	22.83	18.83	1.21	4.73	3.40	0.81	4.23
2	19.88	17.49	1.15	4.23	2.71	0.63	4.58
3	20.82	18.81	1.11	18.60	14.06	5.11	3.16
4	14.67	16.49	0.88	3.64	2.90	0.49	6.78
5	17.95	14.32	1.25	2.26	1.60	0.49	3.27
6	18.38	16.55	1.12	3.05	2.37	0.66	3.66
7	16.11	14.67	1.10	1.89	0.88	0.53	1.67
8	19.65	15.22	1.31	3.02	1.40	0.76	1.85
9	19.37	15.50	1.25	3.04	2.22	0.69	3.32
10	20.22	16.50	1.22	3.37	2.23	0.51	4.36
11	22.42	17.96	1.25	3.52	3.12	0.85	3.68
12	19.41	15.87	1.22	3.10	2.27	0.72	3.30
13	19.96	16.15	1.23	4.76	2.55	0.60	4.24
14	18.62	16.06	1.16	3.62	2.42	0.75	3.23
15	19.40	15.77	1.23	2.41	1.83	0.36	5.08
16	19.18	14.71	1.32	3.12	1.84	0.51	3.57
17	19.02	13.37	1.85	3.35	2.74	0.63	4.50
18	19.45	16.45	1.18	3.71	2.96	0.50	6.02
19	20.40	15.48	1.31	3.24	2.44	0.57	4.54
20	19.13	17.30	1.12	3.17	2.35	0.62	4.39
21	18.77	16.95	1.10	3.37	2.61	0.59	4.41
22	21.89	16.91	1.29	3.76	2.56	0.75	3.43
Mean	19.43	16.24	1.22	4.04	2.88	0.82	3.97
CD (p=0.05)	NS	NS	NS	1.878	1.467	0.784	2.054
CV (%)	14.45	12.30	25.60	28.17	30.86	57.70	31.42

Higher pulp to seed ratio is a desirable character for table purpose jamun and for breeding quality fruits. The data presented in table 3 revealed wide variation in biochemical composition of the fruits of all the 22 accessions. TSS content varied from 11% in Acc-10 to 16.40% in Acc-20. Titrable acidity was found to be maximum in Acc-4. The TSS: acid ratio ranged from 22.77 to 47.60 in the selected accessions. Total sugars were estimated to be highest in Acc-22, while lowest was in acc-8. The sugar: acid ratio also showed considerable variability in the accessions which ranged from 25.12 in Acc-4 to 36.70 in Acc-21. Ascorbic acid content was estimated to be the highest in Acc-22 and lowest in Acc-11. Based on the overall physio-chemical studies conducted for the consecutive two years, it may be inferred that the accession-21 and accession-22 are promising genotypes which may be considered for further utilization.

Table. 3. Bio - chemical attributes jamun accessions

Accession No.	TSS (%)	Acidity %	TSS: Acidity ratio	Ascorbic Acid (mg/100g)	Total sugar (%)	Sugar: Acid ratio
1	13.07	0.34	37.89	36.39	12.06	35.07
2	14.20	0.35	40.56	42.82	11.81	33.88
3	12.23	0.38	32.61	51.34	11.86	31.48
4	11.23	0.50	22.77	39.48	12.49	25.12
5	12.47	0.45	27.77	33.75	11.77	26.30
6	12.37	0.39	31.46	35.68	12.35	31.35
7	12.43	0.46	27.58	36.01	12.07	26.61
8	12.27	0.38	32.25	43.50	11.47	30.12
9	13.00	0.38	33.88	41.37	11.78	30.62
10	11.00	0.43	25.70	35.65	11.48	26.77
11	11.70	0.37	31.78	27.36	12.63	34.30
12	12.63	0.47	26.91	41.30	11.60	24.80
13	12.57	0.44	28.73	38.96	13.07	29.81
14	12.23	0.40	30.66	37.90	13.41	33.56
15	12.53	0.36	34.51	36.74	12.29	33.88
16	12.30	0.36	34.25	40.70	12.63	34.67
17	13.43	0.42	32.01	36.75	11.57	27.65
18	12.23	0.38	32.60	41.26	12.27	32.61
19	12.03	0.36	33.61	49.76	12.17	34.18
20	16.40	0.35	47.60	55.72	11.79	34.37
21	15.97	0.34	46.82	45.39	12.50	36.70
22	15.60	0.41	39.63	57.70	14.49	36.31
Mean	12.90	0.40	33.25	41.16	12.25	31.37
CD (p=0.05)	1.058	0.073	7.870	7.519	1.013	5.882
CV (%)	4.97	11.16	14.36	11.08	5.01	11.38

A total of 10 ISSR and 15 RAPD primers (Table 4) were used to infer the genetic diversity among 22 different genotypes of *Syzygium cuminii*. Among 10 ISSR primers used, 5 primers produced amplification, a total of 503 DNA fragments were amplified and produced a total of 38 amplicons across 22 accessions of which 24 amplicons were found to be polymorphic with 63.15% level of polymorphism. The average numbers of polymorphic bands per primer was 4.8. IS 12 primer gave maximum polymorphic band of 77.77 % (Fig.1). The clustering pattern of 22 accessions based on UPGMA analysis with Jaccard's similarity coefficient varied from 0.58 to 1.00 (Fig.2). ISSR dendrogram obtained two main clusters that are Cluster I and Cluster II. Cluster I has two sub Cluster IA Cluster IB. Cluster IA has the accessions of South Andaman. Whereas cluster IB has the accessions of South Andaman and Nicobar. Cluster II has two sub clusters IIA Cluster IIB. Cluster IIA has two sub clusters IIA_1 and IIA_2. Cluster IIA_1 having the accessions of South Andaman, North and Middle Andaman whereas Cluster IIA_2 having the accessions of South Andaman, North and Middle Andaman. The cluster IIB having the accessions of South Andaman and Nicobar. South Andaman accessions showed 66 % similarity with Middle & North Andaman and 70 % with those from Nicobar.

Table. 4. Details of fourteen primers

Sl.No.	Sequence name	Primer sequence 5' to 3'	GC content (%)
		RAPD	
1	OPF 4	GGTGATCAGG	60
2	OPF 8	GGGATATCGG	60
3	OPA 10	GTGATCGCAG	60
4	OPF 1	ACGGATCCTG	60
5	OPA 6	GGTCCCTGAC	70
6	OPQ 4	AGTGCGCTGA	60
7	OPA 9	GGGTAACGCC	70
		ISSR	
1	IS12	GAGAGAGAGAGAGAGAA	47.05
2	IS18	CACACACACACACACAG	52.94
3	IS32	CACACACACACACACACG	55.55
4	IS39	TGTGTGTGTGTGTGTGAA	44.44
5	IS13	CTCTCTCTCTCTCTCTT	47.05

Fig.1. Gel picture of ISSR primer (IS 12)

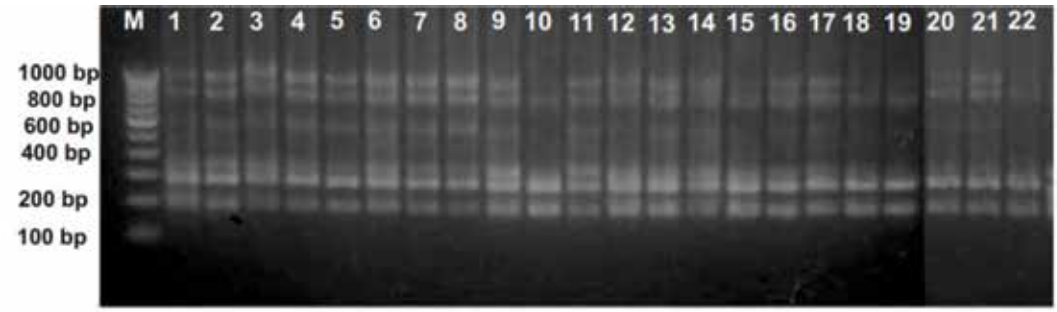
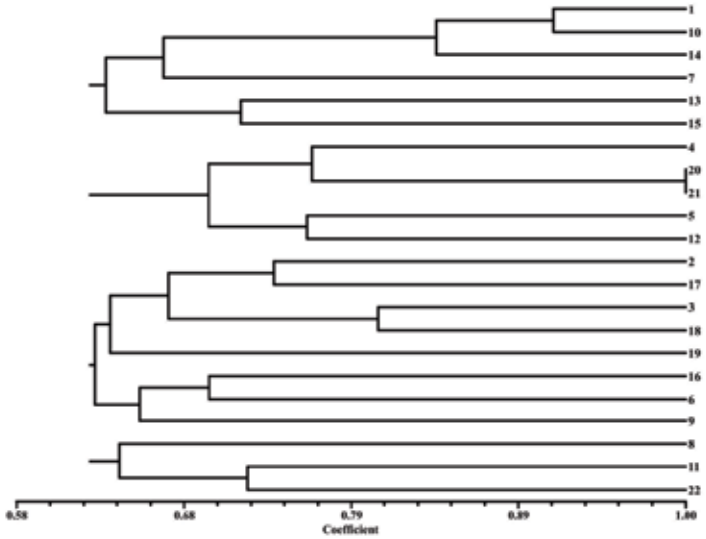


Fig. 2. Dendrogram of ISSR primers for Jamun accessions



Similar results were obtained from 7 RAPD primers which produced a total of 715 DNA fragments. Out of 52 amplified bands, there were 31 polymorphic bands of 7 primers and the average percentage of polymorphism was 59.61. The average numbers of polymorphic bands per primer was 4.42. OPF 1 primer gave maximum polymorphic band of 75 % (Table 5 and 6). The clustering pattern of 22 accessions based on UPGMA analysis with Jaccard's similarity coefficient from 0.63 to 0.81 (Fig.3). RAPD dendrogram obtained two main clusters viz., Cluster I and Cluster II. Cluster I has two sub clusters IA and IB. The Cluster IA comprised of accessions from South Andaman, North and Middle Andaman and Nicobar whereas the Cluster IB exhibited the accessions of North and Middle Andaman. The cluster II has two sub clusters viz., cluster IIA and IIB. The IIA has two sub clusters viz., cluster IIA_1 and cluster IIA_2. The Cluster IIA_1 include the accessions of South, North and Middle whereas the IIA_2 has the accessions of Nicobar and cluster IIB has the accessions of South Andaman. South Andaman accessions displayed 74 % similarity with North and Middle Andaman accessions and 76% with those from Nicobar. In the present study IS12 and OPF 1 primers gave more polymorphism compared to other primers. Shakya e*t al*., (2010) also reported that in *Syzygium cuminii* the primers OPZ9 and OPA12 recorded the highest polymorphic bands. Assessment of genetic diversity of cultivated crop plants is very important to select proper genotypes for any hybridization programme. It is an important tool of crop improvement programme and can also be helpful in protecting and documenting the biodiversity of various agro- economically important crops of a region. RAPD and ISSR marker systems are routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences (Escribano *et al*., 2004; Iqbal *et al*., 1997). Overall comparison of ISSR and RAPD was indicative of greater efficiency of ISSR and RAPD markers for diversity assessment. Based upon the cluster analysis of combined data of RAPD and ISSR, two main clusters (Cluster I and Cluster II) were obtained from the dendrogram. The Jaccard's similarity coefficient of UPGMA analysis of twenty two accessions varied from 0.61 to 0.83 (Fig.4). The Cluster I contained two sub clusters *viz*., clusters IA and IB wherein the sub cluster IA had two sub clusters IA_1 and IA_2. Sub cluster IA_1 was having the accessions of South Andaman whereas Sub Cluster IA_2 was having the accessions of South Andaman, North and Middle Andaman and Nicobar. The Sub cluster IB contained the accessions from South Andaman. The cluster II was having the accessions from North and Middle Andaman and Nicobar. South Andaman accessions exhibited 61 % similarity with Middle & North Andaman accessions and 63 % similarity with Nicobar. Among the Nicobar accessions 83 % similarity was observed. In the present study 22 accessions collected from the same geographical regions normally grouped together and depict very high similarity (63.15% with ISSR and 59.61% with RAPD). This high genetic similarity suggested that genotypes arose more or less from common sources or there is more gene flow with in the agro ecological zone. High gene flow is attributed to random mating followed by very little selection. These findings are in accordance with Shakya *et al.,* (2010) in *Syzygium cuminii*, and Kingdom *et al.,* (2007) in *Annona* spp. Selection of germplasm on the basis of the *Syzygium cuminii* dendrogram can be used for collection of appropriate parental material to improve fruit yield. In this study, accessions 20, 21 1nd 22 collected from Nicobar Island showed maximum diversity compared to other Island accessions as revealed by morpho - biochemical as well as DNA marker based variability analysis. Hence, these accessions can be utilized to generate sufficient genetic variability for crop improvement.

Table. 5. Combined details of fourteen primers (07 RAPD + 5 ISSR) and amplified bands in 22 *Syzygium cumini* accession

Primers	Number of total bands	No. of Polymorphic bands	No. of Monomorphic bands	% of polymorphic bands	Total number of bands amplified	Average number of bands	PIC
			ISSR				
IS12	9	7	2	77.77	96	10.66	0.331
IS18	7	4	3	57.14	103	14.71	0.272
IS32	7	4	3	57.14	102	14.57	0.266
IS39	8	5	3	62.50	105	13.12	0.253
IS13	7	4	3	57.14	97	13.85	0.120
			RAPD				
OPF 4	10	6	4	60	122	12.2	0.390
OPF 8	7	5	2	71.42	108	15.42	0.356
OPA 10	6	3	3	50	88	14.66	0.213
OPF 1	8	6	2	75	80	10	0.282
OPA 6	7	3	4	42.85	110	15.71	0.319
OPQ 4	6	3	3	50	88	14.66	0.217
OPA 9	8	5	3	62.5	119	14.87	0.223

Table. 6. A comparative list showing different markers details (RAPD, ISSR and RAPD +ISSR) obtained for the 22 *Syzygium cumini* accessions

Primers	RAPD	ISSR	RADP + ISSR
Number of primers used	7	5	12
Total number of polymorphic bands	31	24	55
Total number of monomorphic bands	21	14	35
Total number of bands	52	38	90
Total number of bands amplified	715	503	1218
Percentage polymorphism (%)	59.61	63.15	61.11
Average number of bands/ primer	7.42	7.6	7.5
Average number of polymorphic bands/primer	4.42	4.8	4.85

This study is the first report of *Syzygium cuminii* using molecular markers in association with physico chemical traits in tropical Bay Islands. Two classes of markers *viz*., RAPD and ISSR were important for the determination of genetic diversity and relationships amongst 22 accessions of *Syzygium cuminii* collected from Andaman and Nicobar Islands, India. Characterization based on the ISSR molecular markers was found to be more efficient than RAPD markers with common geographical backgrounds. Furthermore, ISSR markers were superior to RAPD markers with respect to the percentage-detection of polymorphism and discrimination of the more related genotypes of Islands.

Fig. 3. Dendrogram of RAPD primers for Jamun accessions

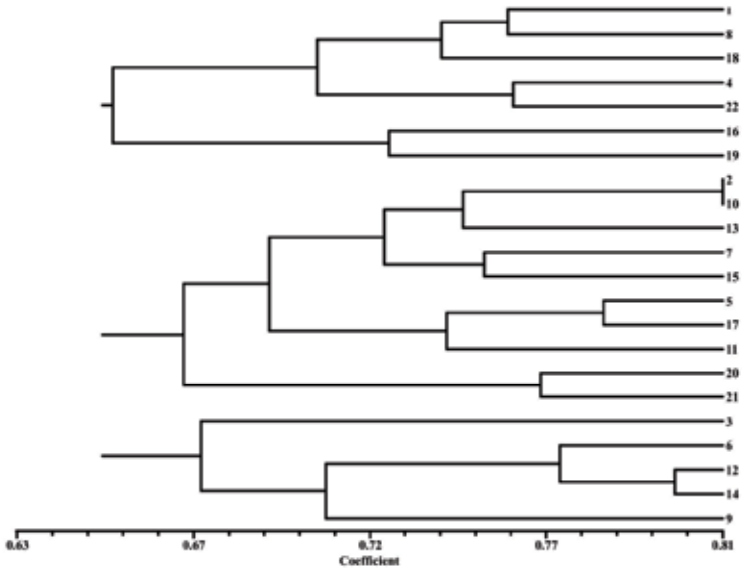
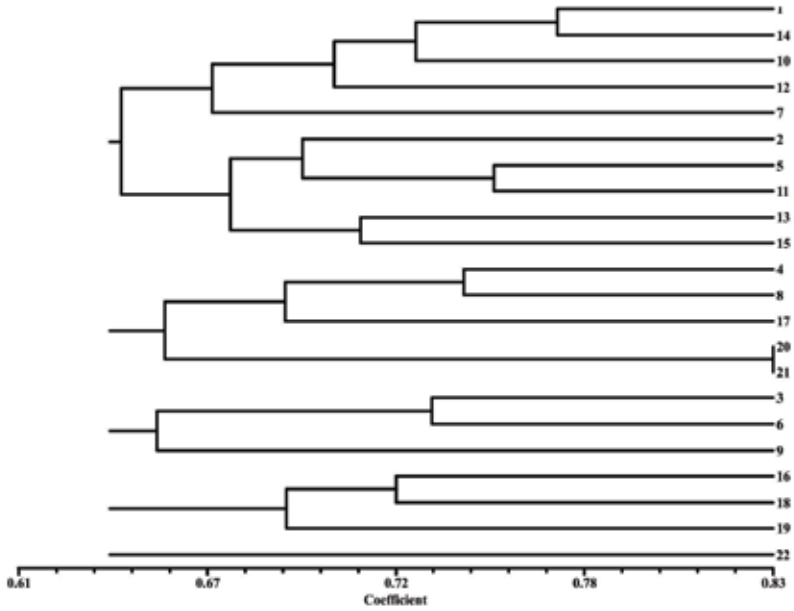


Fig. 4. Combined dendrogram of RAPD + ISSR primers for Jamun accessions



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