Research Paper :

Genetic diversity among different *Ricinus communis* genotypes for Fusarium wilt through molecular markers

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SUMMARY

Castor (*Ricinus communis* L.) is an important plant for production of industrial oil. The systematic evaluation of the molecular diversity encompassed in castor offers an efficient means of exploiting the heterosis in castor as well as for the development of disease resistant varieties. Three DNA-based molecular marker techniques, *viz.,* random amplied polymorphism DNA (RAPD), simple sequence repeat (SSR) and inter simple sequence repeat (ISSR), were used to assess the genetic diversity in castor genotypes. Out of 27 RAPD, 15 SSR and 17 ISSR primers screened, a total of 29 polymorphic primers (11 RAPDs, 8 SSRs and 10 ISSRs), were used in this study. Amplification of genomic DNA of 9 genotypes, using RAPD analysis, yielded 83 fragments, of which 64 were polymorphic, with an average PIC value of 0.59. Number of amplified fragments with RAPD primers ranged from 2 t o 13, with the size of amplicons ranging from 100 to 3000 bp in size. The polymorphism ranged from 54.54 to 100.0, with an average of 81.81per cent. Similarly, SSR analysis yielded 10 fragments, of which 5 were polymorphic, with an average PIC value of 0.14. The 10 ISSR primers produced 71 bands across 9 genotypes, of which 54 were polymorphic, with an average PIC value of 0.25. The number of amplified bands varied from 3 to 15, with size of amplicons ranging from 100 to 2500 bp. The percentage of polymorphism using ISSR primers ranged from 38 to 100.0, with an average of 76.02 per cent. Clustering of genotypes within the groups was not similar when RAPD, SSR and ISSR derived dendrograms were compared, whereas, the pattern of clustering of the genotypes remained akin in RAPD and combined data of RAPD, SSR and ISSR. The similarity coefficient ranged from 0.61 to 0.98, 0.66 to 1.00, 0.62 to 0.83 and 0.65 to 0.85 with RAPD, SSR, ISSR, and combined dendrogramme, respectively. Knowledge on the genetic diversity of castor can be used to future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

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Castor plant is a tropical plant that belongs to family Euphorbiaceous and is grown for its non-edible oilseed. In scientific terms, this plant is also termed as *Ricinus communis*. It is cultivated around the world because of the commercial importance of its oil. India is the world's largest producer of castor seed and meets most of the global demand for castor oil. India contributes 750,000 tons annually, and accounting for over 60 per cent of the entire global production.

Castor is an industrial oilseed crop. Because of its almost unlimited industrial applications, castor oil enjoys tremendous world demand. India is the world's top producer and exporter of castor. Wilt (*Fusarium oxysporum* f.sp.*ricini*) is the major disease of castor (*Ricinus communis*) in India and it can cause 80–100% crop damage. Fusarium wilt is a common fungal vascular wilt disease. The pathogen that causes Fusarial wilt is *Fusarium oxysporum*. It affects a wide variety of hosts of any age and generally produces symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off.

Characterization of the genetic basis of resistance to *F. oxysporum* in castor germplasm based on molecular marker-assisted selection strategies will allow easier effective

incorporation of resistance genes into widely-cultivated castor cultivars that are currently commonly susceptible to wilt. Assessment of genetic variation for resistant gene using molecular markers appears to be an attractive alternative to the conventional method and can also aid in management and conservation of biodiversity. A large number of polymorphic markers are required to measure genetic diversity for resistant gene in a reliable manner (Santalla *et al*., 1998). This limits the use of morphological characters and isozymes, which are limited in number or lack adequate diversity in castor. Further, isozyme analyses have inherent disadvantages such as limited numbers of markers, and are often less effective due to their inconsistency and sensitivity to short-term environmental fuctuations (Lowrey and Crawford, 1985; Soltis *et al*., 1992; Crawford *et al*., 1995; Francisco *et al*., 1996; Esselman *et al*., 1997; Lesica *et al*., 1998). DNA-based molecular analysis tools are ideal for germplasm characterization for resistant gene.

Among the various DNA-based markers, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have been used earlier. These markers elucidate the phylogenetic relationships among various lines, for their efficient use in breeding and genetic resource management. These methods, however, involve the use of expensive enzymes, radioactive labeling, and are cumbersome and hence, appear unsuitable. Random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers on the other hand, require only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster. RAPD has proven to be quite efficient in detecting genetic variations and used for diversity assessment and for identifying germplasm in a number of plant species (Welsh and McClelland, 1990; Gwanama *et al*., 2000; Kapteyn and Simon, 2002). ISSR and SSR have been shown to provide a powerful, rapid, simple, reproducible and inexpensive means to assess genetic diversity and identify differences between closely related cultivars in many species (Gonzalez *et al*., 2002). Limited studies have been carried out in castor using molecular markers.

Considering the economic importance and the limited research work done in the area of DNA based molecular marker in castor, the present study was designed to investigate and characterize the genetic diversity present in the Indian genotypes of castor for resistant gene using RAPD, ISSR and SSR markers, with an ultimate aim to identify polymorphic markers that could be exploited for genetic improvement of castor through breeding and marker assisted selection (MAS), as well as, in future germplasm conservation strategies.

MATERIALS AND METHODS

Plant material:

For the present experiment, 09 varieties of castors usually grown in Saurashtra region of Gujarat were selected on the basis of their performance in the sick plot developed for the screening of Fusarium wilt. The list of genotype with their performance in sick lot has been mentioned in Table 1.

Table 1: Castor varieties of *Fusarium* wilt disease

Castor varieties of *Fusarium* wilt disease		
Susceptible	A	JI-399
	B	JI-401
	C	JI-400
Moderate	D	JI-258
	E	JI-380
Resistance	F	JI-368
	G	JI-220
	H	JI-244
	I	JI-357

DNA isolation:

Young and tender leaves were collected from the plant during the month of December and January. Total genomic DNA was isolated from the leaves according to Doyle and Doyle (1990) with some modification. Leaves were ground in liquid nitrogen using mortar and pestle to fine powder. It was then transferred to pre-warmed extraction buffer and incubated at 65°C for 1 h. An equal amount of chloroform: isoamyl alcohol (1:1) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding ¾ volume of isopropanol. After centrifugation, the pellet was washed in 70 per cent ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified by Picodrop (Picodrop Ltd. Cambridge, UK.).

RAPD analysis:

Amplification of RAPD fragments were performed according to Williams *et al.* (1990) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA) (Table 2). The amplifications were performed in a 25 µl reaction volume containing 50 ng of template DNA, 2.5 mM of each dNTPs (Promega, USA), 20 pM of primer (Operon Technologies Inc., USA), 1.5

Table 2 : Parameter of RAPD primers used for genetic analysis

Primer sequence	Primer sequence	Tm	GC (%)	Allele size	Number of allele (A)	Polymorphic band (B)	Polymorphic % (B/A)	PIC value
OPA 03	AGT CAG CCA C	34.3	60	600 –1200	3	3	100	0.3456790
OPA 05	AGG GGT CTT G	32.6	60	500 –2000	4	4	100	0.4646465
OPA 15	TTC CGA ACC C	34.2	60	100 –2000	11	8	72.72	0.6419753
OPD 02	GGA CCC AAC C	36.2	70	100 –1000	8	7	87.50	0.9703704
OPD 08	GTG TGC CCC A	40.1	70	500 –2000	7	7	100	0.9691358
OPE 03	CCA GAT GCA C	32.6	60	300 –1000	2	2	100	0.4881292
OPE 07	AGA TGC AGC C	35.1	60	200 –1200	11	6	54.54	0.6201330
OPE 15	ACG CAC AAC C	36.9	60	100 –1500	13	11	84.61	0.3915344
OPE 16	GGT GAC TGT G	31.8	60	900 –3000	7	5	71.42	0.4993141
OPF 13	GGC TGC AGA A	35.6	60	100 –1500	8	5	62.50	0.1934156
OPH 13	GAC GCC ACA C	38.7	70	200 –2000	9	6	66.66	0.9135802
	Total				83	64	---	---
	Mean				7.54	5.81	81.81	0.5907191

U of Taq DNA polymerase (Bangalore Genei, India) and 1X PCR buffer [Tris (pH 9.0), KCl, 15 mM $MgCl_2$], (Bangalore Genei, India). The amplification reaction consisted of an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 1min at 94°C, 1 min at 36°C and 2 min at 72 °C followed by a final extension step at 72 °C for 7 min. in an Applied Biosystem 2720 thermal cycler.

ISSR analysis:

ISSR amplification reactions were carried out in (Table 3) 25 µl volume containing, 40 ng of template DNA, 2.5 mM of each dNTPs (Promega, USA), 20 pM of primer (Applied Biosciences), 1.5 U of Taq DNA polymerase (Bangalore Genei, India) and 1x reaction buffer (Banglore Genei, India). The amplification reaction consisted of an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 1min at 94°C, 1 min at a specific annealing temperature depending on the Tm values of the primers (Table 3), and 2 min at 72 °C followed by a final extension step at 72 °C for 7 min.

SSR analysis:

The SSR amplification was carried out (Table 4) with 40 ng of template DNA, 2.5 mM of each dNTPs (Promega, USA), 10 pM of each forward and reverse primer (Applied Biosciences), 1.0 U of Taq DNA polymerase (Bangalore Genei, India) and 1x reaction buffer (Banglore Genei, India) in a total volume of 25 µl. The amplification reaction consisted of an initial denaturation step at 94°C for 10 min., followed by 35 cycles of 45 Sec. at 94°C, 45 Sec. at a specific annealing

Table 3 : Parameter of ISSR primers used for genetic analysis

ISSR primers	Primer sequence	Tm	GC (%)	Allele size	Number of allele (A)	Polymorphic band (B)	Polymorphic % (B/A)	PIC value
PO-02	ATCGATCGATCGATCG	57.9	50	600 – 1000	03	03	100	0.345679
D-12	GAGAGAGAGAGACG	39.2	57	160 – 600	07	02	28.57	0.098765
D-14	CACCACCACGC	45.5	73	220 – 1500	11	11	100	0.386083
I0-04	CACCACCACGA	45.5	73	200 – 1500	06	04	66.66	0.213992
HB-13	GAGGAGGAGGC	40.1	73	350 – 1300	06	06	100	0.411523
ISSR-06	CTCCTCCTCGC	58.6	73	200 – 1300	05	02	40	0.158025
HB-14	GTGGTGGTGGC	42.0	73	300 – 700	03	02	66.66	0.131687
HB-15	AGCAGCAGCAGCAGCGC	43.5	70.5	200 – 2500	15	12	80	0.283128
ISSR-09	GAGAGAGAGAGAGAGAGAAAG	48.8	46.6	130 – 900	10	07	70	0.167901
HB-12	GAGAGAGAGAGAGAGAT	45.5	47.3	100 – 800	05	05	100	0.345679
Total					71	54	760.23	2.54246
Mean					7.1	5.4	76.023	0.254246

temperature depending on the Tm values of the primers (Table 3), and 1.5 min at 72 °C followed by a final extension step at 72 °C for 7 min.

Agarose gel electrophoresis:

Amplified products were separated in 1.5, 1.8 and 2.5% agarose gel for RAPD, ISSR and SSR respectively. A constant voltage of 50 - 100 was provided for 1 - 2 hr. DNA fragments were visualized under UV light. The patterns were photographed using Gel-doc system (Bio- Rad) and stored as digital pictures. The reproducibility of the amplification was confirmed by repeating each experiment three times.

Data collection and analysis:

The relatedness of DNA samples was assessed by comparing RAPD, ISSR and SSR fragments of DNA separated according to their sizes and the presence/absence of shared fragments. The banding patterns obtained from RAPD, ISSR and SSR were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated using the programme NTSYS-PC (Rohlf, 1992). Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) method by SAHN clustering function of NTSYS-pc. Relationships among castor varieties were graphically represented in the form of dendrograms. The cophenetic correlation analysis was carried out using the COPH function of NTSYS-pc. In this method, dendrogram and similarity matrix were correlated to find the goodness-of-fit of the dendrogram constructed based on similarity coefficients.

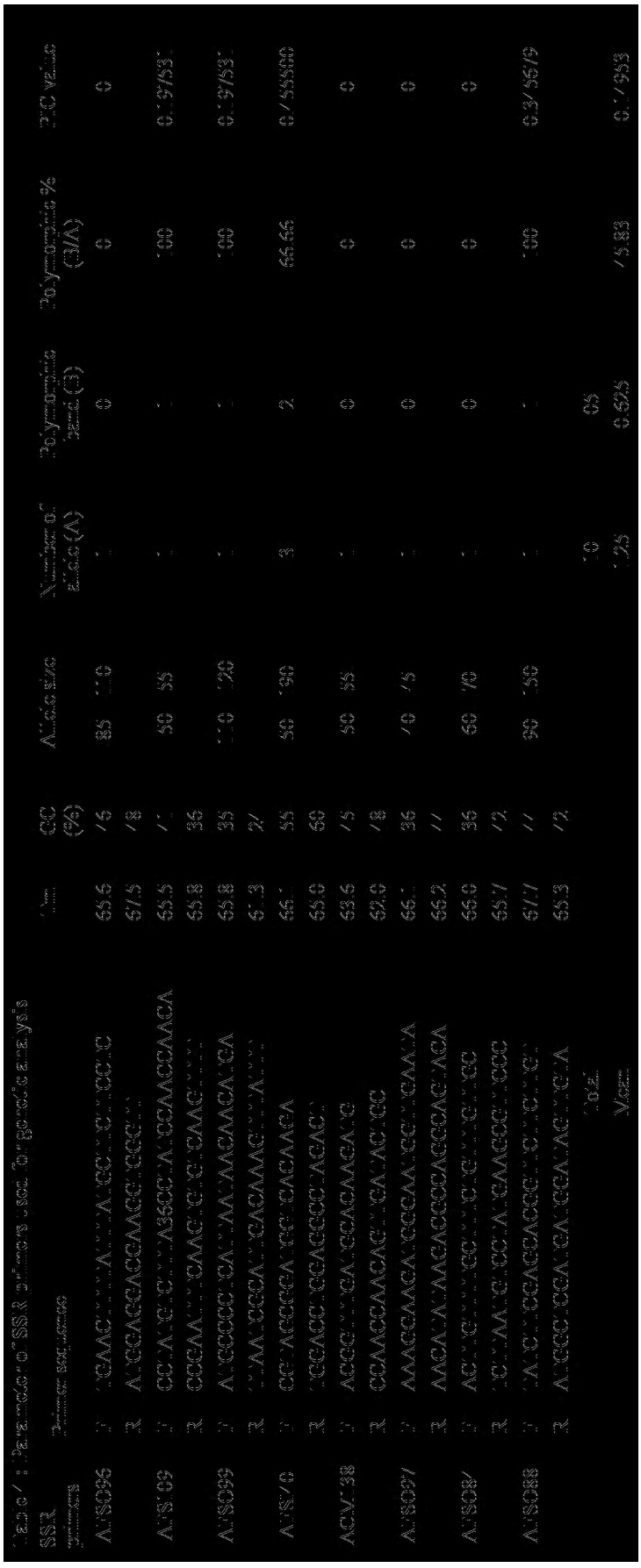
Polymorphism information content (PIC) value calculation:

To measure the informativeness of the different markers, the Polymorphism Information Content (PIC) of each marker was calculated according to following formula (Salem *et al*., 2008).

$$PIC = 1-(Pi^2 + Qi^2)$$

where,

n = total number of allele detected for a locus of



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a marker

Pi = frequency of the *i*th allele in the set of 08 varieties.

Qi = n-Pi

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been presented under following heads:

RAPD analysis:

A total 27 RAPD primers were screened and out of which 11 primers which responded with good resolution and band reproducibility were used in the final analysis in the present study. PCR amplification of DNA, using 11 primers for RAPD analysis, produced total 83 DNA fragments that could be scored in all cultivars. All the selected primers amplified DNA fragments across the 9 castor cultivars studied, with the number of amplified fragment varying with size ranging from 100 to 3000 bp. (Table 2), out of the 83 amplified fragments, 81.81 per cent fragments were polymorphic, with an average of 5.81 polymorphic bands per primer. The PIC value ranged from 0.1934156 to 0.9703704.

A dendrogram based on UPGMA analysis grouped the 9 castor cultivars into two main clusters A and B with Jaccard's similarity coefficient ranging from 0.610 to 0.989 (Fig. 1). Cluster A was again divided into two sub-clusters A1 and A2. A1 had two genotypes JI-399 and JI-401. Sub-cluster A2 also had two genotypes JI-400 and JI-258. Cluster B was divided into two sub-clusters B1 and B2. Sub-cluster B1 had only one genotype JI-380, while Sub-cluster B2 was further divided into clusters B2a and B2b. B2a comprised only one genotype JI-368,

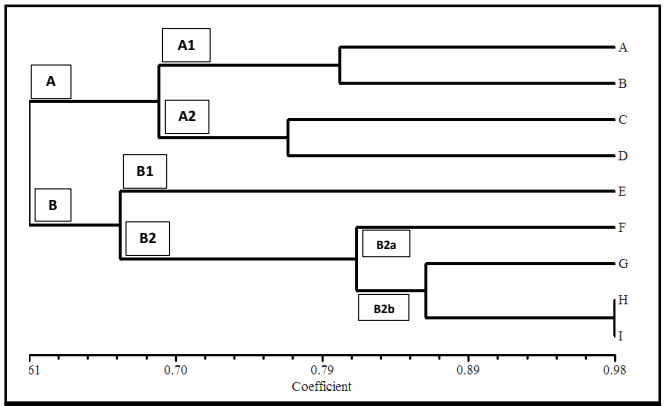

Fig. 1 : Dendogram depicting the genetic relationship among the castor cultivars based on the RAPD data. {JI-399(A), JI-401(B), JI-400(C), JI-258(D), JI-380(E), JI-368(F), JI-220(G), JI-244(H) and JI-357(I)}

while B2b had three genotypes JI-220, JI-244 and JI-357. The highest similarity index value of 0.989130 was found between JI-244 and JI-357, while the least similarity index value of 0.61was found between JI-244 and JI-401 (Fig. 1).

ISSR analysis:

Total 17 ISSR primers were screened and 10 primers which resulted in better polymorphism were selected for further analysis. Thus, ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. The 10 ISSR primers produced total 71 bands across 09 cultivars, of which 54 were polymorphic. The size of fragments ranged from 100-2500 bp. Average number of bands per primer was 5.4. PIC values varied from 0.098 to 0.411 with an average of 0.254 (Table 3).

A dendrogram based on UPGMA analysis of nine castor cultivars with ISSR data is shown in Fig. 2. Jaccard's similarity coefficient ranged from 0.620 to 0.830. Dendrogram generated by ISSR molecular data gave two main clusters, clusters A and B. Cluster A was divided into two sub-clusters A1 and A2. Cluster A1 further divided into A1a and A1b. A1a grouped in to JI-399 and JI-401 genotypes, while A1b grouped in to JI-400, JI-258 and JI-368. Cluster A2 was grouped in to A2a and A2b. A2a grouped in to JI-220 and JI-357 genotypes, while A2b had one genotype JI-244. Cluster B comprised only one genotype JI-380. The highest similarity index value of 0.830 was found between JI-400 and JI-258. While the least similarity index value of 0.620 was found between JI-220 and JI-380.

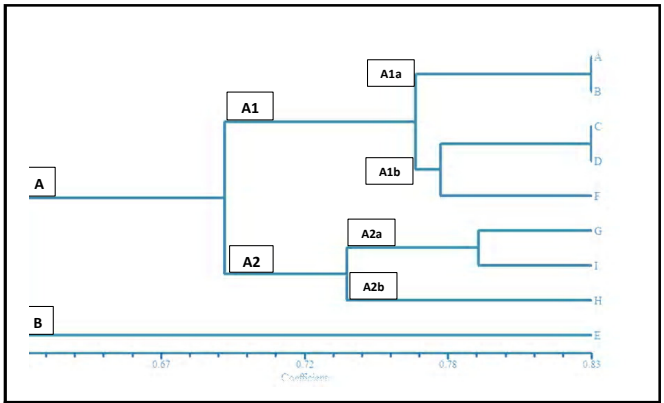

Fig. 2 : Dendogram depicting the genetic relationship among the castor cultivars based on the ISSR data

SSR analysis:

Total 15 SSR primers were screened and out of

which 08 primers responded with more than six markers and which gave fingerprints with good resolution and band reproducibility were used in the final analysis in the present study. The 8 SSR primers produced total 10 bands across 9 cultivars, out them 05 was polymorphic. Average number of bands per primer was 0.625, PIC values varied from 0 to 0.455 with an average of 0.14953 (Table 4).

Dendrogram generated by SSR data gave two main clusters, A and B. Cluster A had only one line JI-399. Cluster B was divided into two sub-clusters B1 and B2. B1 further divided into two sub-clusters B1a and B1b. B1a contained three genotypes JI-401, JI-368 and JI-244, while B1b containing JI-400 and JI-258. Similarly B2 further divided into two sub-cluster B2a and B2b. B2a contained two genotypes JI-380 and JI-357, while B2b contained only one genotype JI-220(Fig. 3). The highest similarity index value of 1.0000 was found between JI-400 and JI-258. While the least similarity index value of 0.66 was found between JI-399 and JI-220.

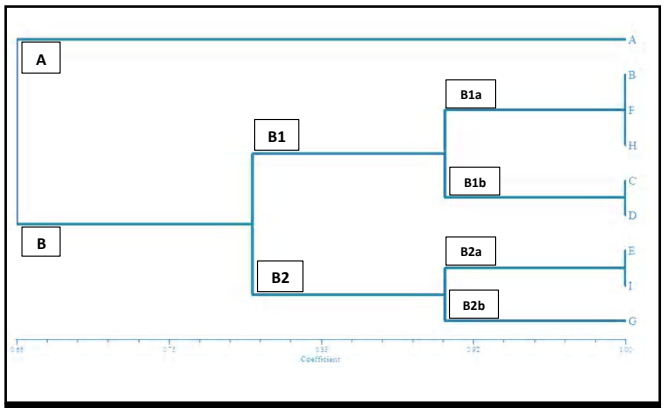

Fig. 3 : Dendogram depicting the genetic relationship among the castor cultivars based on the SSR data

Combined RAPD, ISSR and SSR analysis:

The RAPD, ISSR and SSR data were combined for UPGMA cluster analysis of 09 castor cultivars. The UPGMA dendrogram obtained from the cluster analysis of SSR, ISSR and RAPD data is shown in Fig. 4. Similarity coefficient ranged from 0.653 to 0.850. Cluster analysis performed from combining data of markers generated a dendrogram that separated the cultivars into two distinct clusters, cluster A and B. Cluster A again divided into two sub-clusters A1 and A2. A1 having two genotypes JI-399 and JI-401. A2 also having two genotypes JI-400 and JI-258. Cluster B divided in to B1 and B2. B1 having one genotype JI-380, while B2 further divided in to B2a and B2b. B2a having one genotype JI-368 while B2b having three genotypes JI-220, JI-244 and JI-357 (Fig. 4).

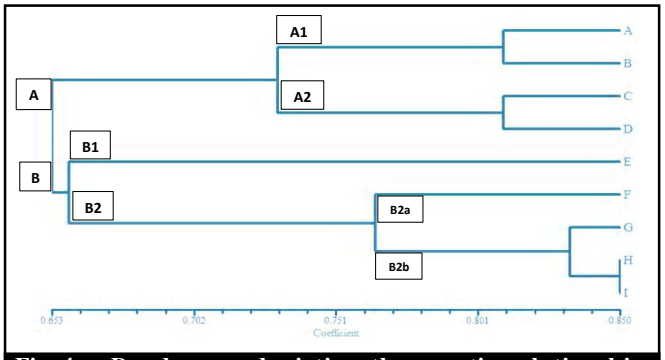

Fig. 4 : Dendogram depicting the genetic relationship among the castor cultivars based on the combined RAPD, ISSR and SSR data

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