

Research Article

Analysis of genetic diversity among different *Ricinus communis* genotypes for macrophomina root rot through RAPD and microsatellite markers

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ABSTRACT

Due to Macrophomina root rot the production of castor has been affected at large. Molecular diversity encompassed in castor offers an efficient means of exploiting disease resistant varieties. Three DNA-based molecular marker techniques, viz., random amplified polymorphism DNA (RAPD), inter simple sequence repeat (ISSR), and simple sequence repeat (SSR) were used to assess the genetic diversity in castor genotypes. Out of the 25 RAPD, 24 ISSR and 10 SSR primers screened, a total of 29 polymorphic primers (10 RAPDs, 10 ISSRs and 9 SSRs), were used in this study. Amplification of genomic DNA of 8 genotypes, using RAPD analysis, yielded 92 fragments, of which 72 were polymorphic, with an average PIC value of 0.29. Number of amplified fragments with RAPD primers ranged from 4 to 13, with the size of amplicons ranging from 100 to 2650 bp in size. The polymorphism ranged from 54.54 to 100.0, with an average of 79.54 per cent. The 10 ISSR primers produced 97 bands across 8 genotypes, of which 55 were polymorphic, with an average PIC value of 0.50. The number of amplified bands varied from 7 to 13, with size of amplicons ranging from 100 to 1600 bp. The percentage of polymorphism using ISSR primers ranged from 28.57 to 85.71, with an average of 56.29 per cent. Similarly, SSR analysis yielded 16 fragments, of which 11 were polymorphic, with an average PIC value of 0.87. Clustering of genotypes within the groups was not similar when RAPD, ISSR and SSR derived dendrograms were compared, whereas, the pattern of clustering of the genotypes remained akin in RAPD and combined data of RAPD, ISSR and SSR. The similarity coefficient ranged from 0.60 to 0.86, 0.63 to 0.80, 0.57 to 1.00 and 0.64 to 0.83 with RAPD, SSR, ISSR, and combined dendrogram, respectively. Knowledge on the genetic diversity of castor can be used to future breeding programmes 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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INTRODUCTION

Castor (*Ricinus communis* L., $2n = 2x = 20$) is a tropical plant that belongs to the Euphorbiaceae family and is grown for its non-edible oilseed. 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. The seeds of castor contain more than 45 per cent oil and this oil is rich (80–90%) in an unusual hydroxyl fatty acid, ricinoleic acid (Jeong and Park, 2009). Castor oil is

the only vegetable oil soluble in alcohol, presenting high viscosity, and requiring less heating than other oils during the production of biodiesel (Jeong and Park, 2009). Due to its unique chemical and physical properties, the oil from castor seed is used as raw material for numerous and varied industrial applications, such as manufacture of polymers, coatings, lubricants for aircrafts, cosmetics, etc, and for the production of biodiesel (Jeong and Park, 2009). With more than 95 per cent of the world's castor production concentrated in limited parts of India, China, and Brazil (Sailaja *et al.*, 2008), and because of the ever increasing world-wide demand of castor

for industrial use, there is a pressing need to increase the hectareage and productivity of castor.

On the other hand root rot caused by *Macrophomina phaseolina* is causing immense loss in the yield. Higher yields can only be obtained if hybrids with root rot resistant are produced. Conventional method for the identification of resistant gene and there after introducing it in the new hybrids, in the field, are time consuming, laborious, resource intensive and drastically affected by environmental factors, therefore, a technique that is rapid and not affected by environment is needed for assessment of genetic diversity for resistant gene and selection of parental lines for use in disease resistant hybrid development programmes. Identification of a castor germplasm with durable resistance to diseases would provide a reliable option to release new commercial cultivars to dry land or rain fed areas of the world where root rot is a common disease. The release of new germplasm resistant to root rot has been delayed due to a lack of information about the basis of the inheritance of resistance. In addition, only inoculation procedures of low reliability are currently available, and reactions to root rot are significantly influenced by environmental conditions that can mask the original response to the fungus. Thus, errors in reporting root rot resistance in segregate germplasm have been found.

Characterization of the genetic basis of resistance to *M. phaseolina* 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 root rot disease. 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 the 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 fluctuations (Lowrey and Crawford, 1995; Soltis *et al.*, 1992; Francisco *et al.*, 1996; Essilman *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 has 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, 08 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 *Macrophomina phaseolina*. The list of genotype with their performance in sick lot has been mentioned in Table A.

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) method 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 was performed

Sr. No.	Genotype	Location	Phenotypic character for root rot
1.	Jl-355	Junagadh	Susceptible to <i>Macrophomina</i> root rot
2.	Jl-401	Junagadh	Susceptible to <i>Macrophomina</i> root rot
3.	Jl-400	Junagadh	Susceptible to <i>Macrophomina</i> root rot
4.	Jl-244	Junagadh	Susceptible to <i>Macrophomina</i> root rot
5.	Jl-380	Junagadh	Moderately resistant to <i>Macrophomina</i> root rot
6.	Jl-368	Junagadh	Resistant to <i>Macrophomina</i> root rot
7.	Jl-357	Junagadh	Resistant to <i>Macrophomina</i> root rot
8.	SKP-84	S. K. Nagar	Resistant to <i>Macrophomina</i> root rot

according to Williams *et al.* (1990) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA) (Table B). 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 U of Taq DNA polymerase (Bangalore Genei, India) and 10X PCR buffer [Tris (pH9.0), KCl, 15 mM MgCl₂], (Bangalore Genei, India). The amplification reaction consisted of an initial denaturation step at 94°C for 5 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 5 min. in an Applied Biosystem 2720 thermal cycler.

ISSR analysis:

ISSR amplification reactions were carried out in 25 µl volume containing 40 ng of template DNA, 2.5 mM of each dNTPs (Promega, USA), 20 pM of primer (Applied Biosciences) (Table C), 1.5 U of Taq DNA polymerase (Bangalore Genei, India) and 1x reaction buffer (Bangalore Genei, India). The amplification reaction consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 1 min at a specific annealing temperature depending on the T_m values of the primers (Table C), and 2 min at 72°C followed by

a final extension step at 72°C for 5 min.

SSR analysis:

The SSR (Table D) amplification was carried out 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 (Bangalore Genei, India) in a total volume of 25 µl. The amplification reaction consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 45 Sec. at 94°C, 45 Sec. at a specific annealing temperature depending on the T_m values of the primers, and 1.5 min at 72°C followed by a final extension step at 72°C for 5 min.

Agarose gel electrophoresis:

Amplified products were separated in 1.5, 1.8 and 2.5 per cent agarose gel for RAPD, ISSR and SSR, respectively. A constant voltage of 50 - 100 was provided for 1 - 2 hrs. 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.

Primers	Primer sequence	T _m	Molecular weight range (bp)	Total number of bands	Polymorphic band (B)	Polymorphic % (B/A)	PIC value
OPA 03	5'-AGT CAG CCA C-3'	34.3	600-2650	4	3	75	0.242188
OPA 15	5'-TTC CGA ACC C-3'	34.2	100-2000	13	9	69.23	0.271635
OPD 02	5'-GGA CCC AAC C-3'	36.2	100-1800	13	13	100	0.338942
OPD 08	5'-GTG TGC CCC A-3'	40.1	550-2000	9	9	100	0.28125
OPE 03	5'-CCA GAT GCA C-3'	32.6	200-1300	4	4	100	0.390625
OPE 07	5'-AGA TGC AGC C-3'	35.1	250-1250	11	6	54.54	0.272727
OPE 15	5'-ACG CAC AAC C-3'	36.9	150-1800	12	10	83.33	0.346354
OPE 16	5'-GGT GAC TGT G-3'	31.8	150-2400	10	6	60	0.250000
OPF 13	5'-GGC TGC AGA A-3'	35.6	100-1500	10	7	70	0.31875
OPH 13	5'-GAC GCC ACA C-3'	38.7	200-2000	6	5	83.33	0.270833
	Total			92	72	---	---
	Mean			9.2	7.2	79.54	0.29833

ISSR primers	Primer sequence	Tm	Allele size	Number of allele (A)	Polymorphic band (B)	Polymorphic % (B/A)	PIC value
D12	GAGAGAGAGAGACG	39.2	180-900	13	11	84.61	0.22
D14	CACCACCACGC	45.5	250-1400	12	8	66.66	0.69
D24	CACACACACACACG	46.2	200-1500	9	5	55.55	0.47
HB12	CACCACCACGC	45.5	300-1300	8	4	50	1.00
HB13	GAGGAGGAGGC	40.1	100-450	9	7	77.77	0.38
HB14	CTCCTCCTCGC	42.0	120-1600	9	3	33.33	0.38
HB15	GTGGTGGTGGC	43.5	250-900	13	4	30.76	0.38
17899B	CA CA CA CA CA CA GG	44.7	280-1300	10	5	50	0.59
IS 15	GTGTGTGTGTGTAT	42.8	650-1600	7	6	85.71	0.59
ISSR 6	GAGAGAGAGAGAGAGAAAAG	58.6	200-1300	7	2	28.57	0.38
	Total			97	55	---	---
	Mean			9.7	5.5	56.29	0.50

SSR primers	Primer sequence	Tm	Allele size	Number of allele (A)	Polymorphic band (B)	Polymorphic % (B/A)	PIC value
AFSO96	F TGAAC TTTATTTATGCTTCTTCCTC	65.6	85-110	2	1	50	0.5625
	R ATGGAGGACGAAGGTGGGTT	67.5					
AFS111	F TGTTTAATGGACTTTCAATGCCTGT	66.1	75-700	2	2	100	0.6875
	R GCATTA AAAATGAAGAAATCCCGAAG	66.1					
AFS109	F CCTATGCTTTA36CCTATCCAACCAACA	65.5	50-55	1	1	100	0.875
	R CCGAATTTCAAGTGTGTCAAGTTTT	65.8					
AFSO99	F ATGCCCTCATTAATAACAACATGA	65.8	110-120	1	1	100	0.875
	R TTAATCGCATTGACAAAAGTTATTT	61.3					
AFS140	F CGTAGCGGATGGTCACAAGA	66.1	50-190	4	3	75	0.53125
	R TGGACCTGGAGGCCTAGACT	65.0					
ACM 138	F ACGGTTTGATGCACAAGATG	63.6	50-55	1	0	0	1
	R CCAACCAACAGTTGATACTGC	62.0					
AFSO97	F AAAGGAAGATGGGAATGGTTGAATA	66.1	40-45	1	1	100	0.875
	R AACATATAAGACGCCAGGCAGTACA	66.2					
AFSO84	F ACTTGTTTGTCTTCTGTTTGTTC	66.0	60-70	1	0	0	1
	R TCTTAATGTCCTATGAAGCGTCCC	65.7					
AFSO88	F TATCTTCGAGCACGGTCTCTTGT	67.7	90-150	3	2	75	0.583333
	R ATGGCTCGATGATGGATAGTTGTA	65.3					
	Total			16	11	-----	-----
	Mean			8	1.375	75	0.8736

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):

$$PIC = 1 - (P_i^2 + Q_i^2)$$

where,

n = total number of allele detected for a locus of a marker

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

Q_i = n - P_i

RESULTS AND DISCUSSION

The experimental findings of the study have been presented in the following sub heads:

RAPD analysis:

Total 25 RAPD primers were screened and out of which 10 primers responded well with good number of markers. PCR amplification of DNA, using 10 primers for RAPD analysis produced total 92 DNA fragments that could be scored in all cultivars. All the selected primers amplified DNA fragments across the 8 castor cultivars studied, with the number of amplified fragment varying with size ranging from 100 to 2650 bp (Table B). Of the 92 amplified fragments, 79.54 per cent fragments were polymorphic, with an average of 7.2 polymorphic bands per primer. The PIC value ranged from 0.242 to 0.390.

A dendrogram based on UPGMA analysis, grouped the 8 castor cultivars into two main clusters A and B with Jaccard's similarity coefficient ranging from 0.60 to 0.86 (Fig. 1). Cluster A was grouped into two sub-clusters A1 and A2. Sub-cluster A1 which was sub-grouped into A1a and A1b, had genotype JI-355 and JI-401 in A1a and genotype JI-400 and JI-244 in A1b. Sub-cluster A2 comprised of only one genotype JI-380. Cluster B was grouped into two sub-clusters B1 and B2. B1 had only one genotype *ie* JI-368 while B2 had two genotypes JI-357, SKP-84 (Fig. 1). The highest similarity index value of 0.8586957 was found between JI-357 and SKP-84, while the least similarity index value of 0.60 was found between JI-244 and JI-357.

ISSR analysis:

Total 24 ISSR primers were screened and out of which 10 primers responded well with good number of markers. ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. The 10 ISSR primers produced total 97 bands across 8 varieties, of which 42 was monomorphic and 55 was polymorphic and mean

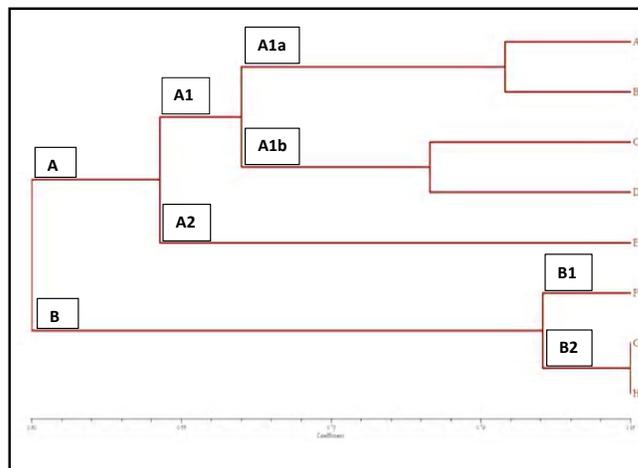


Fig. 1 : Dendrogram depicting the genetic relationship among the castor cultivars based on the RAPD data {JI-355(A), JI-401(B), JI-400(C), JI-244(D), JI-380(E), JI-368(F), JI-357(G), SKP-84(H)}

PIC value of 0.50. The size of fragments ranged from 100 to 1600 bp (Table C).

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 was further grouped into A1a and A1b. A1a had four genotypes JI-355, JI-401, JI-400 and JI-244 while A1b had one genotype JI-368. Cluster A2 had two genotype JI-357 and SKP-84. Cluster B comprised only one genotype *i.e.* JI-380 (Fig. 2). The highest similarity index value of 0.80 was found between JI-401 and JI-400, while the least similarity index value of 0.63 was between JI-401 and JI-380.

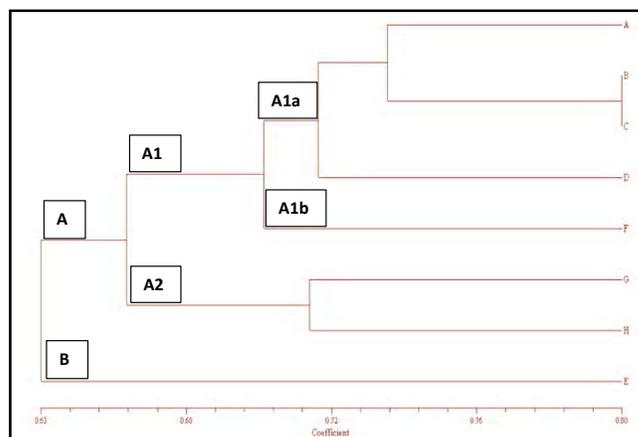


Fig. 2 : Dendrogram depicting the genetic relationship among the castor cultivars based on the ISSR data. Dendrogram is grouped into different clusters and sub-clusters as indicated. {JI-355(A), JI-401(B), JI-400(C), JI-244(D), JI-380(E), JI-368(F), JI-357(G), SKP-84(H)}

SSR analysis:

Total 10 SSR primers were screened and out of which 9 primers responded well with good number of markers. The 9 SSR primers produced total 16 bands across 8 cultivars, out them 11 were polymorphic. Average number of bands per primer was 1.38, PIC values varied from 0.5625 to 0.875 with an average of 0.873 (Table D).

Dendrogram generated by SSR data gave two main clusters, A and B. Cluster A had only one genotype JI-355. Cluster B was divided into two sub-clusters B1 and B2. B1 was further divided into two sub-clusters B1a and B1b. B1a contained three genotypes namely JI-401, JI-357 and JI-244, while B1b contained JI-400. Similarly B2 was also further divided into two sub-cluster B2a and B2b. B2a contained only one genotype *ie* JI-380 while B2b contained two genotypes *ie* JI-368 and SKP-84 (Fig. 3). The highest similarity index value of 1.0000 was found between JI-401 and JI-357. While the

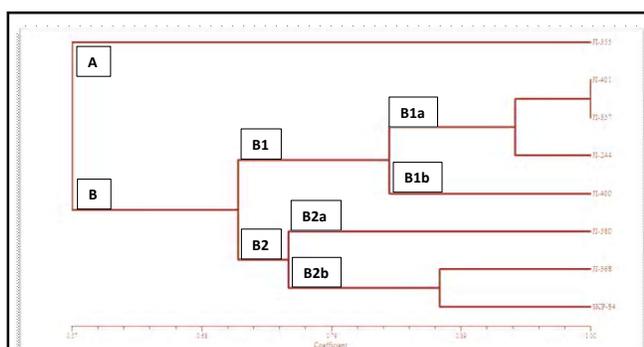


Fig. 3 : Dendrogram depicting the genetic relationship among the castor cultivars based on the SSR data. Dendrogram is grouped into different clusters and sub-clusters as indicated

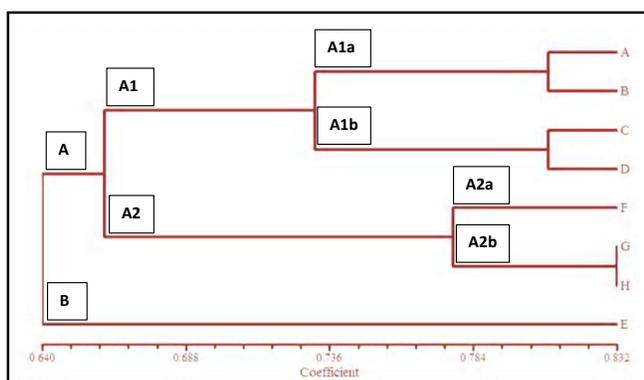


Fig. 4 : Dendrogram depicting the genetic relationship among the castor cultivars based on the combined data of RAPD, ISSR and SSR. Dendrogram is grouped into different clusters and sub-clusters as indicated. {JI-355(A), JI-401(B), JI-400(C), JI-244(D), JI-380(E), JI-368(F), JI-357(G), SKP-84(H)}

least similarity index value of 0.570 was found between JI-355 and JI- 380.

Combined RAPD, ISSR and SSR analysis:

The RAPD, ISSR and SSR data were combined for UPGMA cluster analysis of 08 castor cultivars. The UPGMA dendrogram obtained from the cluster analysis of SSR, ISSR and RAPD data are shown in Fig. 4. Similarity coefficient ranged from 0.589 to 0.832. 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 further divided into A1a and A1b. Both A1a and A1b had two genotypes each namely JI-355 and JI-401 and JI-400 and JI-244, respectively. A2 was divided into A2a which had single genotype JI-368 and A2b which ended in two genotypes JI-357 and SKP-84. While, Cluster B ended only in single genotype JI-380 (Fig. 4).

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