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Evaluation of hybrid purity with their parents in sorghum (*Sorghum bicolor* L. Monech) by using Rapd and SSR markers

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Abstract
Sorghum is one of the main staple food for the world's poorest and most food insecure people across the semi-arid tropic. It is the second crop after maize in developing high yielding hybrids using cytoplasmic-genetic male sterility system (Arya *et al.,* 2014). In this context, determination of genetic purity is an essential requirement for its commercial success of the hybrid. In present investigation, four sorghum hybrids were screened with RAPD and SSR markers for hybrid confirmation and purity testing. Five RAPD primers and eight SSR markers enabled to assess the purity of hybrid with their parents. Among RAPD, the primer OPA-4 produced maximum of 100% polymorphism while primer OPA-18 showed 71.43% polymorphism and found to be least polymorphic. Also, the PIC values of eight polymorphic SSR markers ranged from 0.44 to 0.77 with an average of 0.58. The microsatellite Sb6-84 was observed to be most informative with the PIC value of 0.77, which significantly determined genetic relatedness among hybrids and parental lines. Sb4-121 alone could confirm three hybrids by producing male parent specific allele. The genetic similarity retrieved on the basis of combined RAPD and SSR data using Dice coefficient was ranged from 0.31 to 0.87 with an average of 0.53. The dendrogram developed through UPGMA clustered the hybrids with their respective male parent revealed similarity among them being the true hybrids.

Keywords: *Sorghum bicolor*, hybrid purity, RAPD, SSR, genotyping, cluster analysis

Introduction
Sorghum [*Sorghum bicolor* (L.) Monech] is a very important high biomass producer C_4 plant and has been considered as a 4F crop for its usage as food, feed, fodder and fuel crop. It is a tropical monocot plant belongs to the family Poaceae and one of the most important crops in Africa, Asia and Latin America (Anglani, 1998) [2]. It is one of the world's leading cereal crops, and 3rd most important cereal after wheat and rice in India. Sorghum being nutritionally important having 89% carbohydrates, low fats *i.e.* 8% and 3% of protein (Anonymous, 2015) [3]. The wide range of genetic diversity of sorghum in morphological and agronomic traits such as high photosynthetic efficiency and adaptive pest resistance ushered the possibility of improving its productivity. Its small genome size (750 Mbp) (Arumuganathan and Earle, 1991) [4] is fully sequenced and makes sorghum an attractive model for functional genomics of C_4 grasses (Paterson, 2008). The advent of molecular assays has given some edge to the resistance breeding. In recent years, polymerase chain reaction (PCR)-based technologies availed an easier way for the plant breeders to detect foreign genes in recombinant introgression lines.

Recently, microsatellites are becoming the markers of choice for fingerprinting and genetic diversity studies in a wide range of living organisms (Gupta and Varshney, 2000) [12] and offers an ideal marker system for hybrid purity testing due to its co-dominant inheritance, occurs in high frequency, locus specificity, distribution throughout the genomes of all higher plants and animals, multi-allelic characters reproducibility and robustness (Arya *et al.,* 2014) [5]. In addition, SSR fingerprints displays a high level of polymorphism which are generally highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity (Bruford *et al.,* 1998) [8].

A correct identification of a hybrid plant may often be difficult when it resembles more to one parent than the other or when new morphological combinations of characters arise from recombination of distinct genotypes or when only few morphological traits are available for the analysis. In this regard, the molecular approach may be useful tool for the assessment of hybrid status and for the correct identification of parental lineages (Cennamo *et al.,* 2002) [9].

Full potential of any hybrid can be realized only by using good quality seeds and hence determination of genetic purity is an essential requirement for its commercial success. In a view of Protection of Plant Varieties and Farmers Right, 2001 it has become more important to characterize the cultivars or hybrids. The molecular markers could also be implicated in identification of specific hybrids (Akhare *et al.*, 2008) [1]. Molecular-based seed purity assay are likely to provide promising alternative for identification, registration and protection of commercial sample (Dongre *et al.*, 2012) [10].

Materials and Methods

Plant materials

The experimental material included in present study consisted of four sorghum (*Sorghum bicolor* L. Moench.) hybrids and their parental lines (Table No. 1). Seeds of these lines were collected from Sorghum Research Station (SRS), Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani (MS), India. The present investigation was carried out at the Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, constituent college under Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani (MS), India.

Table 1: List of sorghum Hybrids and their parental line used in this study

Sr. No.	A Line (Female lines)	Hybrids	R Line (Male lines)
1.	AKMS-14A	CSH-14	AKR-150
2.	MS-27A	CSH-16	C- 43
3.	PMS-28A	CSH-25	KR-210
4.	PMS-71A	SPH-1641	KR-196

DNA Extraction

High quality genomic DNA was isolated from young and fresh leaves (6-8 days old) of sorghum using Cetyl Trimethyl Ammonium Bromide (CTAB) DNA extraction method (Saghai-Maroof, 1984) [15] with some modifications.

RAPD and SSR PCR Programming

The parents and hybrids were amplified using five RAPD and eight SSR primers (Imperical Life Science) listed in Table No. 2. A 25 µl PCR reaction mixture contained 25 ng of genomic DNA, 1X PCR buffer, 0.25 mM dNTP, 1.5mM and 1.7 mM $MgCl_2$ for RAPD and SSR respectively, 1U *Taq* polymerase (Banglore Genei Pvt. Ltd.), and 10 pM primer for RAPD and 10 pM each of forward and reverse primers for SSR. The DNA amplification was carried out in thermal cycler (Eppendorf® AG-22331). The optimized thermo cyclic conditions for RAPD amplification consisted an initial denaturation for 7 min at 94 ºC followed by 40 PCR cycles (denaturing at 94 ºC for 1 min, primer annealing at 36 ºC for 1min and primer extension at 72 ºC for 1 min) whereas, for SSR amplification, an initial denaturation at 94 ºC for 5 min followed by 30 PCR cycles (denaturing at 94 ºC for 45 sec, primer annealing at 58-60 ºC for 45 sec and primer extension at 72 ºC for 1 min). A final extension step of 10 min and 8 min at 72 ºC was carried out for polishing the ends of PCR products in RAPD and SSR markers respectively.

Resolution of PCR product

Amplified PCR products were separated on 1.5% agarose gel (100V for 2.5 hr) using 100 bp DNA ladder (Banglore Genei Pvt. Ltd) as a molecular size standard. The gels stained with Ethidium bromide (10 mg/ml) were visualized and photographed under UV light in gel documentation system (Alphaimager™ 2200).

Data analysis

Seven types of marker patterns as described in earlier reports by Akhare *et al.*, (2008) [1] were considered (Table No. 2), which could help to identify purity of the hybrids. Also, The Polymorphism Information Value (PIC) for SSR markers were calculated as, $PIC=\Sigma\ (1-P^2i)/n$, where n is the number of band positions analyzed in the set of accessions and P^2i is the frequency of i^{th} allele (Nei and Li, 1979) [14]. The data was used for similarity based analysis using the programme NTSYS-pc© (Version 2.02i) developed by Rohlf, 2000. The SIMQUAL programme was used to calculate the DICE coefficient and UPGMA cluster analysis.

Table 2: Seven types of markers observed in hybrids and their parents

Markers Type	Male (M)	Hybrid (H)	Female (F)	Remark
1	+	+	+	Good marker to confirm hybrid of its respective parents (male and/or female)
2	-	+	+	
3	+	+	-	
4	+	-	+	Good markers to identify self and off types
5	-	-	+	
6	+	-	+	
7	-	+	-	Hybrid specific

Results

Hybrid confirmation based on RAPD fingerprint profile analysis

A set of four popular sorghum hybrids and their parental lines were initially screened using 8 random primers, out of which only five primers *viz.*, OPA-4, OPA-13, OPA-18, OPA-19 and OPK-19 revealed to be highly informative, polymorphic and reproducible. The primer OPA-4 could produce maximum of 100% polymorphism while primer OPA-18 showed 71.43% polymorphism and found to be least polymorphic. The average number of bands per primer per genotype ranged between 3.67 and 5.50 with an average of 4.60 bands per primer per genotype.

In hybrid CSH-14 and its parent, OPA-13 generated two male parent specific amplicons of ~1200 bp and ~1700bp. Primer OPA-4 and OPA-19 could produce the scorable male parent specific amplicons ~650bp and ~700 bp respectively. In contrast, OPK-19 generated female parent specific amplicon of ~400bp. OPA-18 produced both female parent specific amplicon (~600 bp) and male parent specific amplicon (~400 bp) thus, confirmed hybrid CSH-14 of its respective parent (Figure 1(a)).

In hybrid CSH-16 and its parent, OPA-4 was able to produce two male parent specific bands (~500bp and ~700bp) and one female parent specific bands of ~1000bp. This revealed CSH-16 as a hybrid of its respective parents. OPA-13 generated two male parent specific amplicons of ~1200 bp and ~1500bp. OPA-18 and OPK-19 generated two male parent specific amplicons (~400bp, ~750bp and ~500bp, ~600bp respectively) and was found to be useful to confirm CSH-16 as true hybrid of its male parent whereas, OPK-19 also produced genotype specific band in male parent *i.e.* C-43 (~1200bp) and female parent *i.e.* MS 27A (~450bp). (Figure 1(b)).

When hybrid CSH-25 was compared with its parents using OPA-4 RAPD primer, it produced female parent specific band

(~750bp) and a hybrid specific amplicon (~250 bp). The primer OPA-13 generated male parent specific band (~1200bp). OPA-18 was found to be good marker to confirm hybrid of its respective parent, which produced two male parent specific (~400bp, ~700bp) and one female parent specific amplicon (~900bp). OPA-18 could also produce a genotype specific amplicon (~750 bp) in female parent *i.e* PMS-28A (Figure 1(c)). OPA-19 could produce two female parent specific amplicons (~1000 bp and ~1200 bp). In contrast, OPK-19 produced two male parent specific amplicons (~550 bp and ~650 bp), which proved the true hybrid with its parents. Therefore, OPA-19 and OPK-19 were found to be useful in assessing the purity of CSH-25. OPK-19 produced a genotype specific amplicon (~300 bp) in male parent.

In hybrid SPH-1641 and its parental combination, OPA-4 generated amplicon specific to female parent (~850bp). OPA-19 could generate two female parent specific amplicons (~1200bp and ~1800bp), which confirmed the SPH-1641 as hybrid of its female parent. (Figure 1(d)). The random primers OPA-13, OPA-18 and OPK-19 were found homozygous for SPH-1641 and its parental combination.

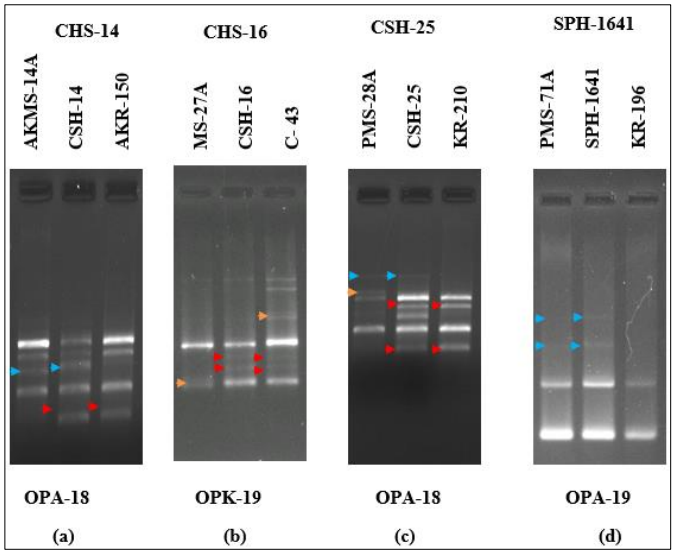


Fig 1: Hybrid confirmation by RAPD markers

Hybrid confirmation based on SSR fingerprint profile analysis

Eight sorghum specific SSR markers showed high polymorphism (96.77%) with 2 to 5 alleles (average 3.75 alleles). The PIC values of eight polymorphic, SSR markers ranged from 0.44 to 0.77 with an average of 0.58 (Table No. 3), which were closer to those reported by Arya *et al*., (2014) [5]. The microsatellite Sb6-84 was observed to be most informative with the PIC value of 0.77 which significantly determined genetic relatedness among hybrids and parental lines (Figure 2a).

The purity of CSH-14 could be assessed with Sb6-57 (produced female parent specific allele, ~300bp) and Sb4-121, Sb6-34 and Sb6-36 (produced male parent specific allele, ~240bp, ~210 and ~190bp respectively) whereas, CSH-14 found to be homozygous with remaining microsatellites.

Sb5-236, Sb4-121 and Sb6-84 revealed to be useful in assessing purity of hybrid CSH-16 by producing male parent specific allele (~160bp, ~160bp and ~210bp respectively).

CSH-25 was heterozygous with Sb6-57 showing presence of both female parent specific (~220bp) and male parent specific (~200bp) alleles (Figure 2(b)). Sb4-121 produced male parent specific allele of ~250bp. In contrast, Sb5-236 and Sb6-84 produced female parent specific alleles (~200bp respectively). Sb6-84 has also produced hybrid specific alleles (~240bp respectively), which could enable development of marker for cultivar identification.

Sb5-236, Sb4-121 and found to be useful to confirm SPH-1641 as a hybrid of its respective female parent by producing female parent specific allele (~190bp and ~230bp respectively). In contrast, Sb6-36 revealed SPH-1641 as a true hybrid of its male parent showing male parent specific allele of ~180bp, ~160bp and ~210bp respectively whereas, SPH-1641 found to be homozygous with remaining microsatellites.

Sb4-121 has produced male parent specific allele in three hybrids (Figure 2c). The primers Sb6-84 and Sb6-36 could produce male specific alleles in two hybrids and confirm the direct introduction of character and identity of the hybrid. Primers, Sb5-236, Sb5-57, and Sb6-34 produced male specific allele in at least one hybrid each.

The four hybrids *viz.*, CSH-14, CSH-16, CSH-25 and SPH-1641 were homozygous with Sb4-72 showing the presence of homozygous allele with female and male parent.

Table 3: Polymorphic alleles generated by SSR markers

Sr. No.	Primer	No. of Alleles	Allele Size (~bp)	PIC Value
1.	Sb6-57	3	170-220	0.59
2.	Sb6-84	5	190-230	0.77
3.	Sb6-42	4	160-200	0.72
4.	Sb4-72	4	200-240	0.76
5.	Sb6-34	3	210-240	0.57
6.	Sb5-236	4	170-180	0.74
7.	Sb4-121	4	220-280	0.65
8.	Sb6-36	2	160-190	0.44
Average		3.75	----	0.58

Clustering based on combined RAPD and SSR data

The similarity matrix generated on the basis of combined RAPD and SSR data was used to develop dendrogram through UPGMA cluster analysis by using NTSYS-pc (Version 2.02i). The genetic similarity retrieved using Dice coefficient was ranged from 0.31 to 0.87 with an average of 0.53 whereas, genetic distance values ranged between 0.13 and 0.69 with an average of 0.47.

The dendrogram (Figure 3) was divided into two major clusters *viz.*, Cluster-I and Cluster-II with three and nine genotypes respectively. Cluster-I included the hybrid CSH-14 and its respective parents. CSH-14 was found nearest to its male parent sharing about 82% similarity. Similarly, in Cluster-II remaining three hybrids were grouped with their respective male parents. Cluster-II was further divided into two subclusters Cluster-IIA and Cluster-IIB. Cluster-IIA included hybrid CSH-16 sharing about 85% similarity with its male parent (C-43) and Cluster-IIB included two hybrids CSH-25 and SPH-1641. SPH-1641 shared maximum genetic similarity (87%) with its male parent. The clustering of the hybrids with their male parents revealed similarity among them being the true hybrids.

Discussion

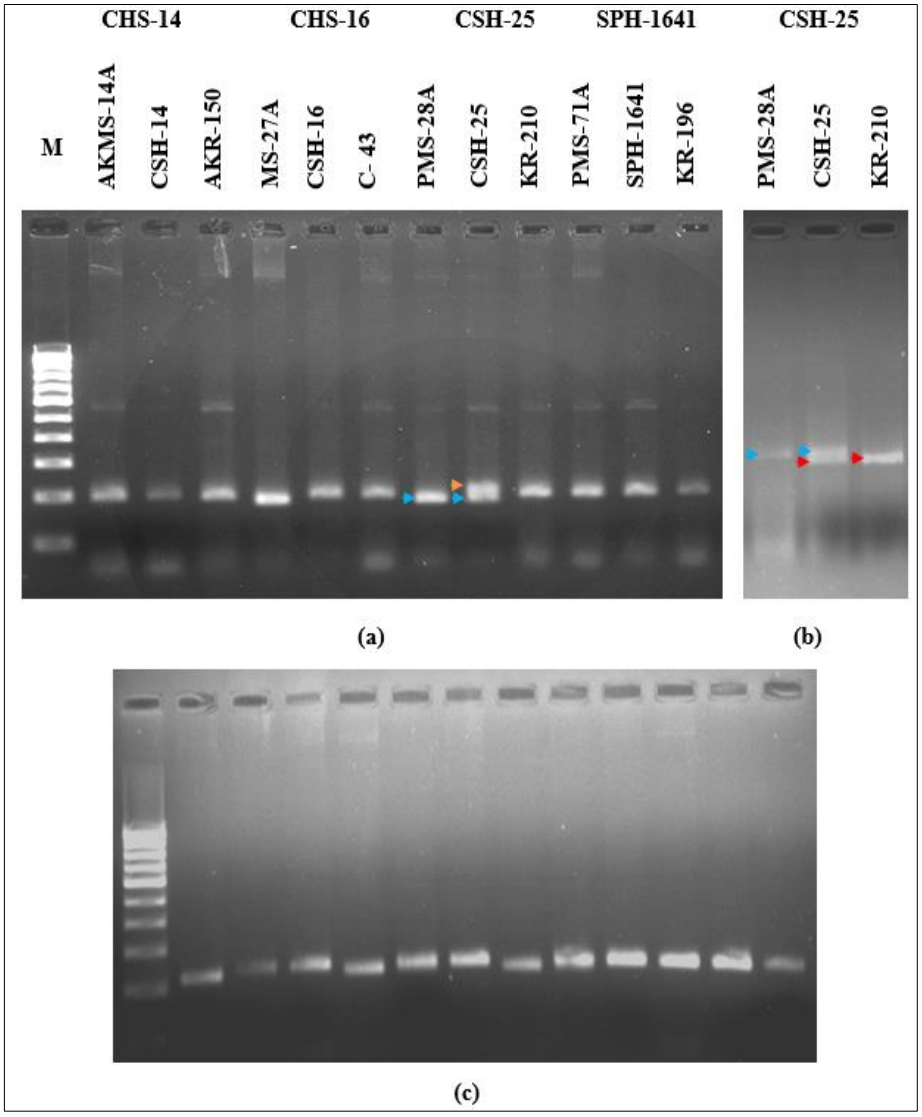
In a systemic hybrid breeding programme it is essential to identify superior parent to exploit the genetic variability for better heterosis development. The use of RAPD technique is easier to identify true hybrids at early stages. Therefore, this technique have been adopted routinely for assessment of

hybrid purity in sorghum (Akhare *et al.*, 2008 [1]; Tabbasam *et al.*, 2006 [17]; Smith *et al.*, 2000 [16]. However, RAPD makers have come under criticism because they lack reproducibility. Therefore, in present study, instead of relying on RAPD, additional reliable microsatellite markers have been used. The results of Dongre *et al.*, (2010) [10] have revealed that using RAPD, ISSR and SSR in combination was more reliable than using the three separately for identification and testing of genetic purity of the hybrids. Similarly, efficacy of RAPD and SSRs for hybrid confirmation and purity testing has been reported (Asif *et al.*, 2009; Benedetti *et al.*, 2000) [6, 7]. Thus, present study was implemented to exploit the utility of molecular marker *viz.*, RAPD and SSR for assessment of hybrid purity in sorghum.

RAPD profiles revealed five RAPD primers *viz.*, OPA-4, OPA-13, OPA-18, OPA-19 and OPK-19 found highly polymorphic and reproducible, generated distinct and polymorphic banding patterns. These primers enabled the assessment of hybrid purity. Similar investigations based on RAPD analysis have been reported in which RAPD markers were successfully employed for parentage verification, hybrid confirmation, cultivar identification and purity testing.

Also, microsatellite markers used in the present investigation exhibited high discriminating ability between sorghum hybrids and their respective parents. Therefore, these primers potentially helped in hybrid confirmation and purity testing. The results were nearly in conformity with earlier reports of Arya *et al.*, (2014) [5] and Smith *et al.*, (2000) [16] with different genotypes and primers. Similar investigations have been performed to identify the ability of SSR markers in demarcation of hybrids and their parental lines as well as genetic purity assessment of hybrids in sorghum (Jessup *et al.*, 2011 and Dweikat, 2005) [13, 11]. In all these studies SSR markers were used for germplasm identification, cultivar fingerprinting, true hybrid identification, genetic purity testing and confirmation of parental lineages and identification of heterotic pattern in hybrids.

The investigation revealed five RAPD (OPA-4, OPA-13, OPA-18, OPA-19 and OPK-19) and eight SSR markers (Sb6-84, Sb6-57, Sb6-42, Sb4-72, Sb6-34, Sb5-236, Sb4-121and Sb6-36) were found useful in assessment of hybrid purity in the present investigation. Also, unique fragments for few of the genotypes represented considerable discrimination between the two genotypes for the given amplified loci, which could enable development of marker for cultivar identification.



a. SSR profile of sorghum hybrids and parental lines with primer Sb6-84
b. SSR profile of CSH-25 and its parental lines with primer Sb6-57
c. SSR profile of sorghum hybrids and parental lines with primer Sb4-121

Fig 2: Hybrid confirmation by SSR markers

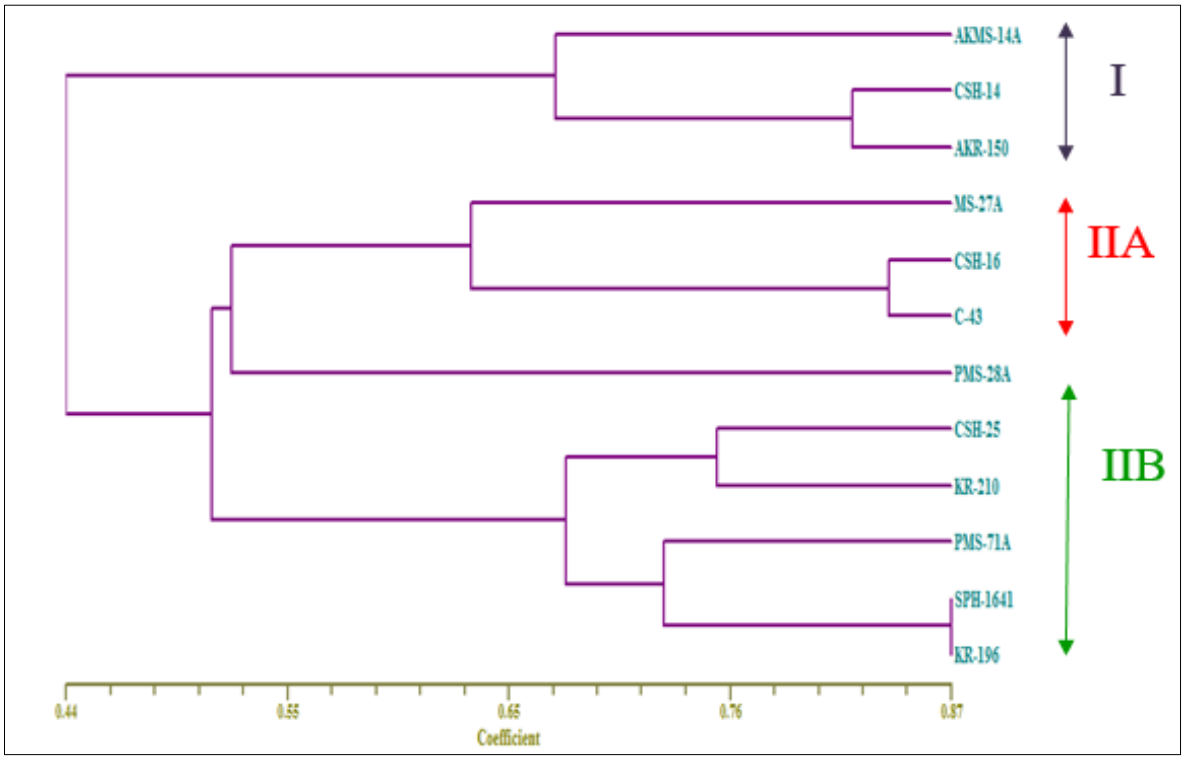


Fig 3: Dendrogram generated by UPGMA analysis based on combined RAPD and SSR data

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