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Molecular Profiling of Selected Cassava (*Manihot esculenta* Crantz) Germplasm Using ISSR and SSR Markers

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Abstract

Root and tuber crops are those plant species that store their food inside their stem or root. They are the staple food for many people in developing countries and also a global source of carbohydrate. Cassava (*Manihot esculenta* Crantz) is a root crop belonging to section Fructicosae, family Euphorbiaceae and of class Dicotyledons. Cassava is a typical diploid species with chromosome number as 2n = 36. Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers provide more sophisticated analysis of genetic diversity, cultivar identification, population genetic structure and other events of evolutionary biology processes. In the present study, the genetic variability among 14 indigenous accessions of cassava germplasm maintained in the field genebank of ICAR-CTCRI was estimated by molecular characterization using six ISSR and nine SSR markers. DNA was extracted from young fresh leaves of cassava using the CTAB method. The genetic distances based on ISSR markers ranged from 0.102941 to 0.231083. Among the 14 cassava accessions, the lowest genetic distance (0.102941) was observed between CI-3 and CI-2 for the 6 ISSR markers and the genetic distances based on SSR markers ranged from 0.00 to 0.283507. The lowest genetic distance (0.00) was observed between CI-3 and CI-2 suggesting that these two genotypes are similar for 9 SSR markers. The value of expected heterozygocity ranged from 0.92 (UBC 807) to 0.98 (UBC 808, UBC 817, UBC 836) and from 0.00 (SSRY 102) to 0.97 (SSRY 45, SSRY 100). The Polymorphism Information Content values ranged from 0.133 (UBC 808, UBC 836) to 0.180 (UBC 845) and from 0.000 (SSRY 102) to 0.500 (SSRY 9, SSRY 147, SSRY 148, SSRY 161) indicating the low to moderate polymorphism for these alleles.The study revealed that only two accessions (CI-161 and CI-296) exhibit 100% similarity by molecular characterization using 6 ISSR and 9 SSR markers among the 14 accessions grouped under a single core group morphologically. Hence, it is concluded that the molecular markers used in the present study can very effectively be used as tools in future for germplasm characterization, variability studies, core collection development and also diversity studies in cassava.

Key words: Cassava, genetic variability, molecular markers, SSR, ISSR, Polymorphism Information Content

Introduction

Cassava (*Manihot esculenta* Crantz) belongs to the family Euphorbiaceae is characterised by lactiferous vessels composed of secretory cells. The centre of origin of cassava was first reported to be Central America including Colombia, Venezuela, Guatemala and Southern Mexico due to the large number of genotypes present there (Sauer, 1952; Roger, 1965). Cassava is regarded as one of the most important staple crops and food for about 800 million people in tropics and sub tropics (FAO, 2007). It contributes to the world food supply in many ways either as eaten roots for human and animals or as industrial starch.

Cassava is generally propagated through stem cuttings, thereby maintaining genotype purity. Under natural conditions as well as in plant breeding, propagation by seed is common and farmers in Africa occasionally use seedlings for their subsequent planting (Silvestre and Arraudeaus, 1983). It generally has a diploid genome (2n = 36), however, some authors have described it as a segmental allotetraploid with basic chromosome number of x = 9 (Jos and Nair, 1979).

Cassava is an efficient producer of carbohydrate under minimal growth conditions like uncertain rainfall, infertile soils and limited inputs encountered in tropical areas that makes cassava an attractive source of food, feed and renewable industrial raw material. It can be stored in the ground for several seasons, thereby serving as a reserve food when other crops fail. Cassava is well adapted to a wide range of environmental stresses. It grows very well in less fertile soil in contrast to many other crops that are highly vulnerable to environmental stresses during critical stages of plant development (Ugorji, 1998). Cassava which is generally propagated vegetatively is one of the major sources of food in Africa (Cock, 1982). The roots which are an excellent source of carbohydrates have very low protein content. In addition, the roots have a high content of cyanogenic glucosides (de Bruijin, 1971) which often require extensive processing before consumption.

Molecular characterization based mainly on DNA molecular markers has been very useful in evaluating the genetic diversity/variability among crop species (Marcio and Mark, 2004; Wang and Bughrara, 2003; Beeching and Marmey, 1993). Molecular markers are regions of DNA that have specific location on a homologous chromosome of two different individuals. DNA based molecular markers are used as tools in taxonomy, physiology, embryology and genetic engineering. Ideal molecular markers have high polymorphism, co-dominant inheritance and frequent occurrence in the genome, high reproducibility, easy and fast assays (Kesawat and Das, 2009).

ISSR is a marker technique, which involves the use of microsatellite sequences as primers in a PCR to generate multilocus markers. It is a simple and quick method that combines most of the advantages of SSRs and AFLP to the universality of RAPD. Ten primers for ISSR were successful in generating reproducible and reliable amplicons for the four imported cassava genotypes identified (Zayed et al., 2012). It can be used to measure genetic distance and to generate molecular profile among selected accessions. ISSR markers have higher reproducibility than RAPD markers which involve PCR amplification of DNA using single primers composed of microsatellite sequences. These primers target microsatellites that are abundant throughout the eukaryotic genome (Tautz and Renz et al., 1984) and evolve rapidly. ISSR markers are mostly dominant markers, though occasionally a few of them exhibit co-dominance. SSRs are the most prominent markers because they are dispersed in all eukaryotic genomes. They are short tandem repeats usually consists of 1-6 bp of nucleotides. They were first referred as microsatellites by Litt and Lutty; 1989 and later as simple sequence repeats (SSR) by Jacob et al.,1991. Microsatellites are hyper-variable and therefore are able to distinguish the closely related plant cultivars (Davila et al.,1998). The SSR markers has become powerful tools for genotype assignment, marker assisted breeding, genetic mapping and diversity assessment (Gupta and Varshney, 2000).

ISSR and SSR markers have been applied to estimate the genetic diversity and relationships in a number of crop species (Bart et al., 2016; Senan and Dhanya, 2013). In the present study, SSR markers were selected based on the previous reports of work in cassava (Mba et al., 2001). Available ISSR markers were also used for studying the genetic diversity of cassava.

Materials and methods

Source of plant materials

Leaf samples of 14 accessions of cassava grouped under a single core group morphologically, maintained in the field genebank of the ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvanathapuram served as source material for the present molecular study (Table 1).

DNA extraction

DNA was extracted from tender leaves of 14 cassava accessions using CTAB method (Doyle and Doyle, 1987). The isolated total DNA was quantified by Nanodrop spectrophotometry and its quality was verified by agarose gel electrophoresis. The quantity of DNA was determined at OD 260

and its purity was calculated from OD at 260/280 ratio.

Table 1. Cassava accessions used for the study

Sl. No.	Material Code	Identity Number
1	C-1	CI -124
2	C-2	CI -161
3	C-3	CI -296
4	C-4	CI -304
5	C-5	CI -373
6	C-6	CI -391
7	C-7	CI -530A
8	C-8	CI -581
9	C-9	CI -615
10	C-10	CI -688
11	C-11	CI -700
12	C-12	CI -703
13	C-13	CI -766
14	C-14	CI -1002

The concentration of DNA was derived using the following formula;

Concentration of DNA (µg/ml) = OD_{260} x dilution factor x 50

Samples were diluted to 10 ng/µl concentration using nuclease free water.

Primer screening

In the present work, total 6 ISSR and 9 SSR primers were used for the analysis. The ISSR primers were UBC-807, UBC-808, UBC-811, UBC-817, UBC-836, UBC-845 and SSR primers used were SSRY 9, SSRY 45, SSRY 100, SSRY 102, SSRY 105, SSRY 147, SSRY 148, SSRY 161, SSRY 181. The list of primers selected, primer sequence and annealing temperatures of both ISSR and SSR primers are given in Table 2, 3 and 4.

PCR amplification

ISSR reaction mixture was prepared as shown in Table 5 and the PCR was carried out in Proflex thermal cycler programmed for an initial denaturation at 94°C for 5 minutes followed by 35 cycles with denaturation at 94°C for 30 seconds, primer annealing at 56°C for 1 min and polymerization at 72°C for 1 min. The final extension at 72°C for 10 min followed by its holding at 4°C.

SSR reaction mixture was prepared as shown in Table 6. Initial denaturation at 94°C for 5 minutes followed by

Table 2. List of ISSR primers used with details

Sl. No.	Primer name	Sequence	Annealing temperature (°C)
1	UBC 807	AGA GAG AGA GAG AGA GT	56.3
2	UBC 808	AGA GAG AGA GAG AGA GC	56.3
3	UBC 811	GAG AGA GAG AGA GAG AC	56.3
4	UBC 817	CAC ACA CAC ACA CAC AA	56.3
5	UBC 836	AGA GAG AGA GAG AGA GYA	56.3
6	UBC 845	CTC TCT CTC TCT CTC TRG	56.3

Table 3. List of SSR primers used with details

Sl. No.	Primer name	Bandsize (bp)	Annealing temperature (°C)
1	SSRY 9	273 – 320	48
2	SSRY 45	150 – 210	48
3	SSRY 100	209 – 273	52
4	SSRY 102	198 – 200	56
5	SSRY 105	145 – 230	57
6	SSRY 147	268 – 302	65
7	SSRY 148	118 – 136	59
8	SSRY 161	128 – 138	67
9	SSRY 181	192 – 216	65

Table 4. List of SSR primers with their sequences

Sl. No.	Primer name	Sequence
1	SSRY 9 (F)SSRY 9 (R)	5' ACA ATT CAT GAG TCA TCA ACT 3'5' CCG TTA TTG TTC CTG GTC CT 3'
2	SSRY 45 (F)SSRY 45 (R)	5' TTG ACA TGA GTG ATA TTT TCT TGA A 3'5' TCC AGT TCA GTA GTT GGC T 3'
3	SSRY 100 (F)SSRY 100 (R)	5' ATC CTT GCC TGA CAT TTT GC 3'5' TTC GCA GAG TCC AAT TGT TG 3'
4	SSRY 102 (F)SSRY 102 (R)	5' TTG AAC ACG TTG AAC AAC CA 3'5' TTG GCT GCT TTC ACT AAT GC 3'
5	SSRY 105 (F)SSRY 105 (R)	5' TCG AGT GGC TTC TGG TCT TC 3'5' CCA ACA TTC GCA CTT TTG GC 3'
6	SSRY 147 (F)SSRY 147 (R)	5' AGA GCG GTG GGG CGA AGA GC 3'5' GTA CAT CAC CAC CAA CGG GC 3'
7	SSRY 148 (F)SSRY 148 (R)	5' CAA TGC TTT ACG GAA GAG CC 3'5' GGC TTC ATC ATG GAA AAA CC 3'
8	SSRY 161 (F)SSRY 161 (R)	5' CCA GCT GTA TGT TGA GTG AGC 3'5' AAG GAA CAC CTC TCC TAG AAT CA 3'
9	SSRY 181 (F)SSRY 181 (R)	5' CAA TCG AAA CCG ACG ATA CA 3'5' GGT AGA TCT GGA TCG AGG AGG 3'

Table 5. ISSR reaction mixture

Ingredients	Stock concentration	Required concentration	Required volume for one reaction (15 µl)
Emerald AMP® GT PCR master mix	2X	1X	7.5µl
Primer	10µM	0.25µM	0.4µl
Template DNA	10ng/µl	40ng	4.0µl
SDW	-	-	3.1µl
Total			15µl

Table 6. SSR reaction mixture

Ingredients	Stock concentration	Required concentration	Required volume for one reaction (15 µl)
Emerald AMP® GT PCR master mix	2X	1X	7.5µl
Primer (F)	10µM	0.25µm	0.4µl
Primer (R)	10µM	0.25µm	0.4µl
Template DNA	10ng/µl	20ng	2.0µl
SDW	-	-	4.7µl
Total			15µl

35 cycles with denaturation at 94°C for 30 seconds, primer annealing at appropriate temperatures as given in Table 2 for 45 seconds and polymerization at 72°C for 1 min. The final extension at 72°C for 20 min followed by its holding at 4°C.

Detection and analysis of PCR products

The amplified products were resolved in a 2% agarose gel together with reference 100bp (GeNei) and 1 kb (NEB) ladder. All PCR reactions were repeated to ensure the reproducibility of bands. The gel was documented in gel documentation system with Genesys software.

Data scoring and analysis

The scoring of the PCR product was done manually, based on presence (1) and absence (0) of bands. UPGMA dendrogram was constructed by using the software 'Darwin'.

Results and discussion

ISSR analysis

A total of 80 scorable bands were produced out of which 67 were polymorphic (83.75%). The percentage of polymorphism ranged from a maximum of 94.11% shown by UBC 836 to a minimum of 66.66% shown by UBC 807 and UBC 811. The ISSR primers used, the number of bands produced by each primer, number of polymorphic and monomorphic bands and percentage polymorphisms are shown in Table 7. The ISSR polymorphism obtained with primer UBC 836 for the selected cassava accessions is shown in the Fig. 1.

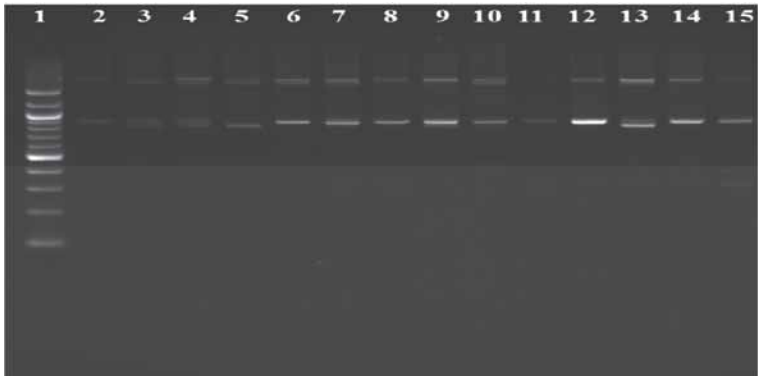

Fig. 1. Agarose gel (2%) profile of the UBC 836 for 14 accessions of cassava, 1-100 bp Ladder, 2-15 accessions.

SSR analysis

A total of 29 scorable bands were produced out of which 21 were polymorphic (72.41%). The SSR primers used, number of bands produced by each primer, number of polymorphic and monomorphic bands and percentage polymorphisms are shown in Table 8. The SSR polymorphism obtained with primer SSRY 105 for the selected cassava accessions is shown in the Fig. 2. Olsen and Schaal (2001) investigated the evolutionary and geographical origins of cassava (220) and the population structure of cassava's wild relatives (33) using five SSR markers. Elias et al., (2004) have used SSR markers to assess the genetic structure of traditional landraces of sweet and bitter cassava collected from five South American sites and also a sample of 38 accessions from a world collection of cultivated cassava. One hundred and sixty local cassava varieties identified as sweet or bitter cassava by traditional farmers from Atlantic forest and

Table 7. PCR analysis using ISSR primers

Sl. No.	Primer	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	Percentage polymorphism
1	UBC 807	6	2	4	66.66
2	UBC 808	17	2	15	88.23
3	UBC 811	15	5	10	66.66
4	UBC 817	13	1	12	92.30
5	UBC 836	17	1	16	94.11
6	UBC 845	12	2	10	83.33

Table 8. PCR analysis using SSR primers

Sl. No.	Primer	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	Percentage polymorphism
1	SSRY 9	2	1	1	50.00
2	SSRY 45	4	1	3	75.00
3	SSRY 100	9	1	8	88.88
4	SSRY 102	1	1	0	0
5	SSRY105	3	0	3	100.00
6	SSRY 147	2	1	1	50.00
7	SSRY 148	2	1	1	50.00
8	SSRY 161	2	1	1	50.00
9	SSRY 181	4	1	3	75.00

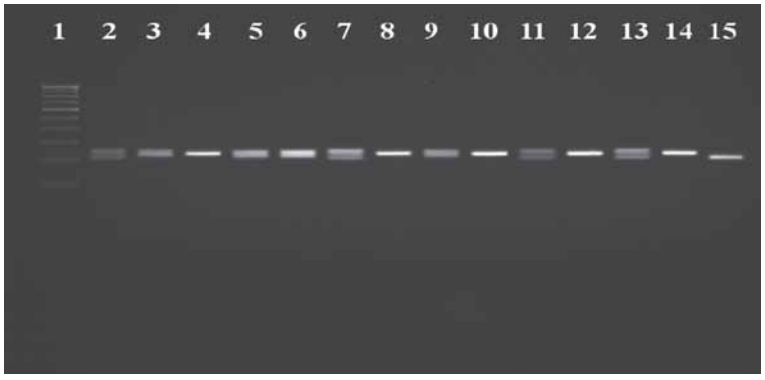

Fig. 2. Agarose gel (2%) profile of the SSRY 105 for 14 accessions of cassava 1-100 bp Ladder, 2-15 accessions

Amazon, Brazil were studied for diversity using SSR markers (Peroni et al., 2007).

Polymorphism Information Content (PIC) and observed heterozygocity of each individual SSR and ISSR allele was calculated according to the formula described by Sharbati-Tehrani et al. (2008) is shown in Table 9 and 10 respectively. The value of heterozygocity ranged from 0.92 (UBC 807) to 0.98 (UBC 808, UBC 817, UBC 836) for ISSR markers indicating high level of heterozygosity. The heterozygosity values for the SSR markers were ranged from 0.00 (SSRY 102) to 0.97 (SSRY 45, SSRY 100) pointing the low to high heterozygosity for these alleles. The PIC values ranged from 0.133 (UBC 808, UBC 836) to 0.180 (UBC 845) and from 0.000 (SSRY 102) to 0.500 (SSRY 9, SSRY 147, SSRY 148, SSRY 161) depicting the moderate to low polymorphism for these alleles. Rahgu et al. (2007) found high level of PIC ranging from 0.842 to 0.987 for SSR markers in 58 cassava accessions. Lekha et al. (2010) observed mean heterozygosity values of 1.3434 for old collections and 1.2252 for new collections of cassava.

Table 9. Polymorphic Information Content and Expected heterozygosity of 6 ISSR primers

Sl. No.	ISSR primer	Average PIC	Expected heterozygosity (He)
1	UBC 807	0.278	0.92
2	UBC 808	0.133	0.98
3	UBC 811	0.153	0.94
4	UBC 817	0.153	0.98
5	UBC 836	0.133	0.98
6	UBC 845	0.180	0.93

Table 10. Polymorphic Information Content and Expected heterozygosity of 9 SSR primers

Sl. No.	ISSR primer	Average PIC	Expected heterozygosity (He)
1	SSRY 9	0.500	0.68
2	SSRY 45	0.210	0.97
3	SSRY 100	0.245	0.97
4	SSRY 102	0.000	0.00
5	SSRY 105	0.245	0.92
6	SSRY 147	0.500	0.71
7	SSRY 148	0.500	0.73
8	SSRY 161	0.500	0.73
9	SSRY 181	0.375	0.93

Cluster analysis

UPGMA cluster analysis using ISSR primers grouped the 14 accessions into three clusters with 2 accessions each shown as in dendrogram (Fig. 3). Cluster I (CI-703, CI-304), Cluster II (CI-688, CI-615), Cluster III (CI- 296, CI-161) and the other 8 accessions (CI-1002, CI-766, CI-581, CI-530A, CI-391, CI-373, CI-700, CI-124) as separate entities.

Cluster analysis using SSR primers also grouped the accessions into three clusters shown as in dendrogram (Fig. 4). Cluster- I with 6 accessions (CI-766, CI-581, CI-530A, CI-296, CI-161, CI-373) was further sub-grouped into cluster IA (CI-766, CI-581) and IB (CI-296, CI-161) in which CI-296 and CI-161 showed 100% similarity. The other 2 accessions CI-530A and CI-373 got separated. Cluster- II with 6 accessions in which 5 accessions (CI-1002, CI-703, CI-688, CI-615, CI-391) form a separate group with CI-304 as separate entity. Cluster- III contain 2 accessions namely CI-700 and CI-124.

Accession wise comparison of ISSR and SSR data is shown in Table 11. The genetic distances based on ISSR markers ranged

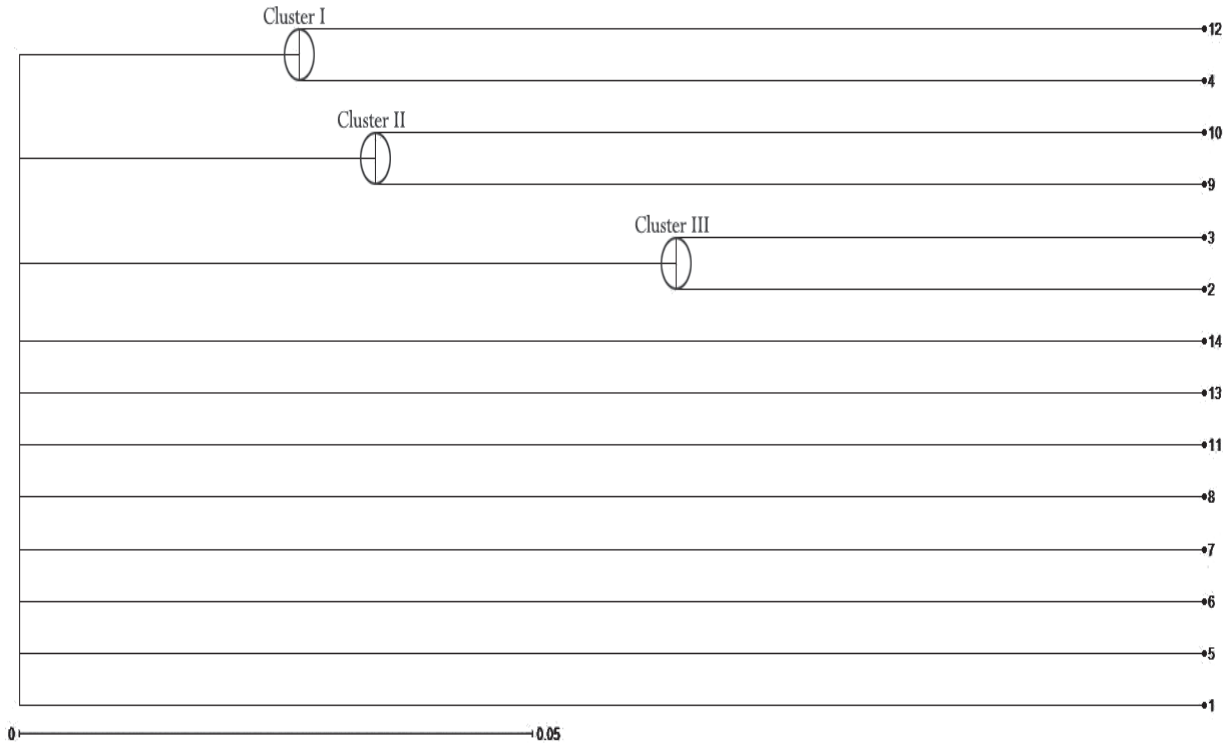

Fig. 3. Dendrogram of 14 accessions of cassava based on 6 ISSR markers

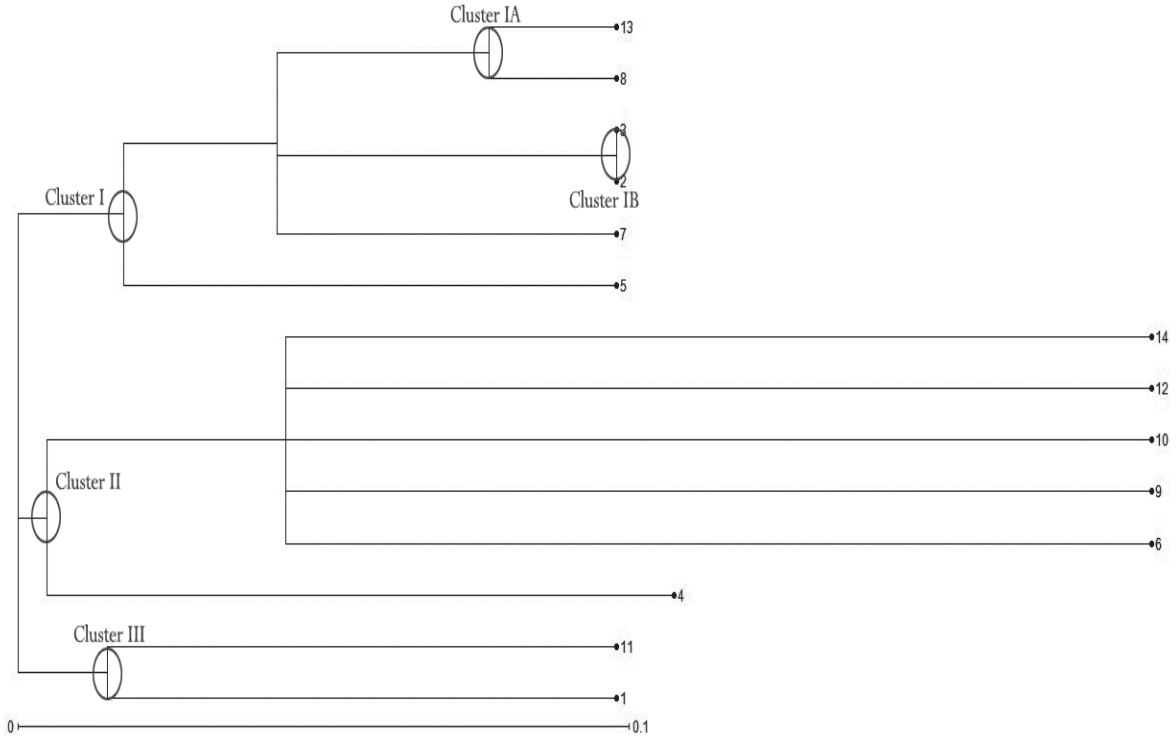

Fig. 4. Dendrogram of 14 accessions of cassava based on 9 SSR markers

Table 11. Accession wise comparison of ISSR and SSR data

Cluster	Accessions (ISSR)	Cluster		Accessions (SSR)
Cluster I	CI-703 and CI-304	Cluster I	I A	CI-766, CI-581
			I B	CI-296, CI-161
		Separate entities		CI-530A, CI-373
Cluster II	CI-688, CI-615	Cluster II		CI-1002, CI-703, CI-688, CI-615, CI-391
	Separate entity	CI-304		
Cluster III	CI-296, CI-161	Cluster III		CI-700, CI-124
Separate entities	CI-1002, CI-766, CI-581, CI-530A, CI-391, CI-373, CI-700, CI-124			

from 0.102941 to 0.231083. Among the 14 cassava accessions, the lowest genetic distance (0.102941) was observed between CI-3 and CI-2. The genetic distances based on SSR markers ranged from 0.00 to 0. 0.283507. The lowest genetic distance (0.00) was observed between CI-3 and CI-2 as they are monomorphic for the 9 SSR markers.

Conclusion

The present study revealed distinct genetic variability among the 14 accessions of cassava by molecular analysis. Among the 14 accessions, only two accessions (CI-296 and CI-161) got grouped in a single cluster in both the groupings with 100% similarity based on the SSR data. The similar accessions maintained as core collections at CTCRI field gene bank gave results differently in molecular studies and hence it is concluded that the molecular markers used in this study can very effectively be used as tools in future for germplasm characterization, variability studies, core collection development and also diversity studies in cassava.

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