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Assessment of cashew species for molecular diversity

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Abstract

Genetic diversity in ten accessions of cashew consisting of three wild species, four genotypes of cultivated species and three inter-specific hybrids was studied using RAPD, microsatellite (SSR) and Isozyme markers. Polymorphic bands generated with 11 RAPD primers, six primer pairs of SSR and Isozymes of six enzymes were used for analysis. A total of 151 bands were obtained with 123 polymorphic bands (81.5%) by combining the markers data. High percentage of polymorphism in markers and an average genetic similarity of 54% suggested high genetic variation and moderate diversity existing among the accessions. The Unweighted Pair Group Method with Arithmetic averaging cluster analysis made on genetic similarity grouped the accessions broadly into three major clusters. In these, *Anacardium pumilum* with its inter-specific hybrids and *Anacardium orthonianum* with its inter-specific hybrid clustered separately and only *Anacardium occidentale* clustered with *Anacardium microcarpum* indicating close affinity between these two species. Even morphologically these two species showed resemblance.

Keywords: Accessions, cashew, diversity, isozymes, RAPD, SSR

Introduction

Cashew (*Anacardium occidentale* L.) is an important nut crop of tropical region cultivated extensively in India, Africa and Brazil. It is the only cultivated species grown in India although many species of cashew are known to exist in the new world. Recently, three wild species namely *Anacardium microcarpum, Anacardium orthonianum* and *Anacardium pumilum* were introduced to India and found inferior to the cultivated species. Further, hybridization of these species with cultivated species was attempted and hybrids were grown. So it has become imperative to understand their genetic relationship before using them in the improvement programme of cultivated type. Here molecular markers provide an important tool for assessing diversity and better utilization of such germplasm. Among the several markers available, Random Amplified Polymorphic DNA (RAPD) (Mneney *et al*., 2001., Dhanaraj *et al*., 2002 and Samal *et al*., 2004), Inter Simple Sequence Repeats (ISSR) (Archak *et al*., 2003), Amplified Fragment Length Polymorphism (AFLP) and Microsatellites (SSR) (Croxford *et al*., 2005) have been found useful in cashew for diversity analysis. Among these, SSR markers are considered robust and efficient as they are highly polymorphic, co-dominant, multiple allelic and highly reproducible in nature. RAPD technique (Williams *et al*., 1990) on the other hand is simple, less expensive and has high throughput capabilities (Waugh and Powell, 1992) yet useful for genetic analysis. In addition to these, Isozyme polymorphism was also used for genetic differentiation of many plants (Arulsekar and Parfitt, 1986 and Bletos *et al*., 2004) but so far has not been tried in cashew. Hence, in this paper the utility of different markers like RAPD, SSR and Isozymes for genetic differentiation of species and inter-specific hybrids in cashew is presented.

Materials and Methods

Plant materials

Ten accessions of cashew consisting of three wild species (*Anacardium pumilum* St. Hilaire, *A. microcarpum* Ducke and *A. orthonianum*), three inter-specific hybrids V-5 (*A. occidentale*) x *A. pumilum*, *A. pumilum* x V-5 (*A. occidentale*) and *A. orthonianum* x

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V-5 (*A. occidentale*) and four genotypes of *A.occidentale* were used for molecular characterization (Table 1). Total DNA was extracted from young cashew leaves following the procedure of CTAB (cetyltrimethylammonium bromide) method of Mneney *et al.* (1997) with slight modification.

SSR markers

The PCR reaction was performed with six selected primer pairs of microsatellite markers developed by Croxford *et al*. (2005) (Table 2). The reaction was carried out in a volume of 25 µl containing 20 ng template DNA, 1X Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTP each, 0.8 µM forward and reverse primer each, 0.75 U of Taq DNA polymerase (M/s. Sigma Aldrich) and autoclaved Milli Q water on a Master cycler gradient (M/s. Eppendorff). The PCR conditions set were: initial denaturation at 94° C for 2 min, cycle denaturation at 94° C for 1min, annealing at 60° C for 1 min, cycle extension at 72° C for 2 min and the cycles were repeated 35 times with a final extension at 72° C for 6 minutes. The PCR products were separated on 8 % denaturing polyacrylamide gel (SE 600 electrophoresis unit – GE Healthcare) stained with silver nitrate and the fragments were sized against the reference bands of 20 bp ladder DNA (Bangalore Genei).

Table 1. Details of species and other accessions used in diversity analysis

NRC Acc. No.	IC.No.	Name of the accession	Type	Salient features
121	249899	Purple type (*Anacardium occidentale* L.)	Mutant	Purple foliage, apple, medium tree, small apple & nut (4.5 g), poor yielding
-	-	V-5 (*A. occidentale* L.) x *A. pumilum*	Inter-specific hybrid	Medium, upright compact, small apple & nut (4 g), moderately yielding
99	249877	VRI-3	An elite variety of Tamil Nadu	Medium, upright compact, medium apple & nut(7 g), high yielding
142	249920	A. *microcarpum* Ducke.	Wild species (Brazil)	Large tree, upright open, medium apple and nut (7.4 g), moderately yielding
-	-	*A. orthonianum* x V5 (*A. occidentale* L.)	Inter-specific hybrid	Large tree, upright compact, small apple & nut (4 g), poor yielding
152	249930	*A. orthonianum*	Wild species (Brazil)	Small to medium tree, upright compact, very small apple & nut(2 g), poor yielding
153	249931	*A. pumilum* St. Hil.	Wild species (Brazil)	Small tree, upright spreading, very small apple & nut (2 g), poor yielding
-	-	*A. pumilum* x V5 (*A. occidentale* L.)	Inter-specific hybrid	Large tree, upright compact, small apple & nut (3 g), poor yielding
360	250138	V5 (*A. occidentale* L.)	Inter-varietal hybrid	Small to medium, upright compact, high yielding, small apple and nut(4 g)
473	-	Kodippady (*A. occidentale* L.)	A local type of Karnataka	Dwarf genotype, poor yielding

RAPD markers

RAPD amplification was carried out with 11 selected primers which were chosen for their consistancy and scorability of bands (Table 2). PCR reaction was performed in a total volume of 25 µl containing 50 ng template DNA, 1.5 units of Taq DNA polymerase, 25 p moles primer, 200 µM each dNTPs, 2.5 mM $MgCl_2$, 1X Taq Buffer A (10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin) (Bangalore Genei, Bangalore) and sterilized milli Q water. The amplification was carried out on a master cycler gradient (Eppendorf, Hamburg, Germany) following: an initial denaturation at 95° C for 2 min, cycle denaturation at 94° C for 1 min, annealing at 35°C for 1 min and cycle extension at 72° C for 2 min. The cycle was repeated 35 times and terminated with a final extension of chain at 72° C for 6 min. The amplified products were held at 4° C and stored at -20° C until they were separated on 1.2 % agarose gels with 1 X TAE buffer and bands were visualized in UV after staining with ethidium bromide.

Isozyme markers

Isozyme extraction was carried out by grinding fresh leaf sample (100 mg) of cashew in chilled 1000 µl extraction buffer of Arulsekar and Parfitt (1986) with freshly added β-mercaptoethanol and a pinch of polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15,000 rpm for 10 min at 4° C and the

Table 2. List of RAPD, SSR and Isozyme markers used in the study

Method	Primer / enzyme/ Locus	Primer Sequence (5'-3')	Total no. of bands	Polymorphic bands		*PIC	MI	Amplicon size (bp)
				No.	%			
RAPD	OPB-20	GGA CCC TTA C	10	8	80.0	0.23	1.81	500-1400
	OPM-10	TCT GGC GCA C	10	8	80.0	0.37	2.96	550-1900
	OPO-01	GGC ACG TAA G	5	5	100.0	0.42	2.12	300-1500
	OPM-18	CAC CAT CCG T	6	5	83.3	0.28	1.40	350-1600
	OPQ-19	CCC CCT ATC A	10	10	100.0	0.35	3.48	600-2000
	OPM-5	GGG AAC GTG T	10	6	60.0	0.19	1.13	300-1200
	OPM-14	AGG GTC GTT C	8	6	75.0	0.29	1.76	650-2100
	OPN-01	CTC ACG TTG G	6	4	83.3	0.24	1.18	875-1900
	OPN-08	ACC TCA GCT C	15	11	73.3	0.26	2.83	300-1550
	OPN-14	TCG TGC GGG T	7	6	85.7	0.24	1.46	410-1000
	OPN-19	GTC CGT ACT G	14	10	71.4	0.28	2.81	500-2000
		Total / Mean	**101**	**80**	**79.2**	**0.29**	**2.08**	
SSR	mAoR3a	F - CAGAACCGTCACTCCACTCC R - ATCCAGACGAAGAAGCGATG	2	2	100.0	0.25	0.50	240-245
	mAoR6c	F - CAAAACTAGCCGGAATCTAGC R - CCCCATCAAACCCTTATGAC	4	4	100.0	0.475	1.90	155-180
	mAoR29c	F - GGAGAAGAAAAGTTAGGTTTGAC R - CGTCTTCTTCCACATGATTC	3	2	67.0	0.20	0.40	950-1000
	mAoR41	F - GCTTAGCCGGCACGATATTA R - AGCTCACCTCGTTTCGTTTC	3	2	67.0	0.32	0.96	170-185
	mAoR42c	F - ACTGTCACGTCAATGGCATC R - GCGAAGGTCAAAGAGCAGTC	3	2	67.0	0.30	0.60	190-205
	mAoR48a	F - CAGCGAGTGGCTTACGAAAT R- GACCATGGGCTTGATACGTC	3	3	100.0	0.487	1.46	170-190
		Total / Mean	**18**	**15**	**83.3**	**0.34**	**0.97**	
Isozymes	AAT	Aspartate aminotransferase	7	6	85.7	0.35	2.08	-
	PER	Peroxidase	6	6	100.0	0.32	1.92	-
	ACP	Acid phosphatase	6	4	66.6	0.30	1.20	-
	EST	Esterase	6	5	83.3	0.30	1.48	-
	PGI	Phospho glucoisomerase	4	4	100.0	0.30	1.18	-
	PGM	Phospho glucomutase	3	3	100.0	0.41	1.22	-
		Total / Mean	**32**	**28**	**87.5**	**0.33**	**1.51**	
		Overall Total / Mean	**151**	**123**	**81.5**	**0.32**	**1.52**	

*PIC - polymorphic information content; MI-marker index

supernatant collected was used for the enzyme assay. Seven enzyme systems namely aspartate amino transferase (AAT, E.C .2.6.1.1), peroxidase (PER, E.C. 1.11.1.7), acid phosphatase (ACP, E.C.3.1.3.2), esterase (EST, E.C. 3.2.1.2), phospho gluco isomerase (PGI, E.C. 5.3.1.9), phospho gluco mutase (PGM, E.C.2.7.5.1) and glucose-6-phosphate dehydrogenase (G-6 PD, E.C.1.1.1.49) were assayed (Table 2). The Isozyme bands were separated on 7.5 % polyacrylamide native gel with Tris-glycine buffer and the gel bands were stained and visualized as detailed by Vallejos (1986).

Data recording and analysis

The binary data matrix made with different markers and accessions were analyzed by treating all the markers as dominant markers. The binary scores made as '1' for presence of band and '0' for absence of band were used for estimating Jaccard's similarity co-efficient and the similarity matrix obtained was used in cluster analysis following the software package of NTSYS-pc Version 2.02 (Rohlf, 1998) (Exeter Software, New York, USA). A dendrogram was constructed using the Unweighted Pair Method with an Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). The confidence limits for the dendrogram groupings were determined by bootstrapping using the Win Boot programme (Yap and Nelson, 1996). The average polymorphic information content (PIC) for each primer was calculated as per the formula given below (Powell *et al*., 1996 and Smith *et al*., 1997)

$$PIC = 1 - \sum_{i=1}^{n} fi^2$$

Where 'fi' is the frequency of the 'i'th allele. The efficacy of each primer was also calculated by Marker Index (MI) which is equal to PIC x No. of polymorphic bands.

The similarity matrix based on RAPD, Isozymes and SSR markers were compared by the MXCOMP routine of NTSYS pc. The normalized Mantel statistic Z (Mantel, 1967) was used to determine the level of association between the matrices.

Results and Discussion

Diversity in accessions was assessed using RAPD, SSR and Isozyme markers alone and by combining all the markers data. RAPD analysis carried out with 11 primers had generated a total of 101 bands, of which 80 bands (79.2%) were polymorphic (Fig. 1a). The number of polymorphic bands varied from 5 (OPO-01; OPM-18; OPN-01) to 11 (OPQ-19; OPN-19) with a mean number of 7.3 polymorphic bands per primer and the size of the amplicons varied from 300-2100 bp. The PIC content of primers varied from 0.19 (OPM-5) to 0.42 (OPO-01) with an average of 0.29 and the MI varied from 1.13 (OPM-05) to 3.48 (OPQ-19) with an average of 2.08 (Table 2). Among the primers used, three primers namely OPO-01, OPM-10 and OPQ-19 were found to be highly informative with high polymorphic information content (0.35-0.42) and marker index (2.12-3.48). The polymorphism generated with 11 RAPD primers alone was sufficient to discriminate all the accessions.

Though DNA markers like RAPD, ISSR and AFLP were used for diversity analysis in cashew (Dhanaraj *et al*., 2002, Archak *et al*., 2003), use of Isozymes in cashew is relatively new. Similarly only limited number of microsatellite markers have been reported in cashew (Croxford *et al*., 2005). SSR analysis carried out with six primer pairs of cashew generated 18 bands, of which 15 bands were polymorphic (83.3%) (Fig. 1b). The number of polymorphic bands varied from 2 to 4 with a mean of 3 bands per primer pair (Table 2). The PIC content of primers varied from 0.2 (mAoR29^c) to 0.487 (mAoR48^a) with an average of 0.34 and MI varied from 0.42 ((mAoR29^c) to 1.90 (mAoR6^c) with an average of 0.97. Primer pairs of mAoR48^a, mAoR6^c and mAoR41 loci were found to be highly informative with high PIC content (0.300-0.487) and MI (0.96-1.90). SSR markers exhibited higher polymorphism than RAPD and almost equal to that of Isozyme markers.

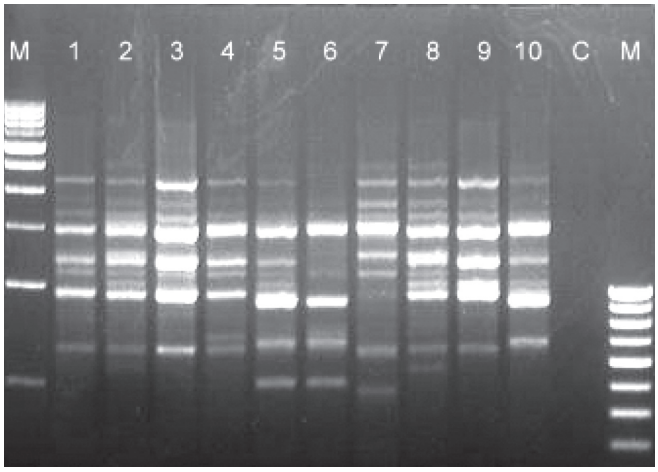

Fig. 1a. RAPD profile of 10 accessions with OPN-19

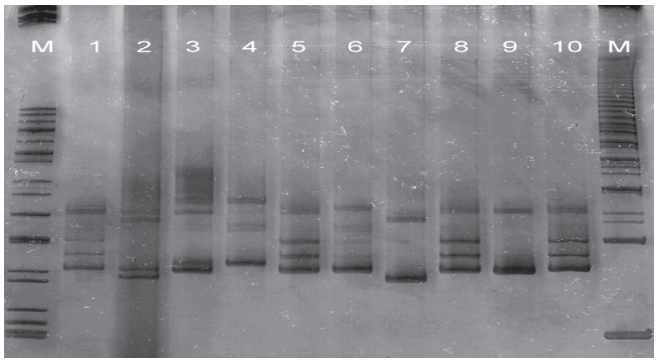

Fig. 1b. Denaturing PAGE profile with SSR primer of locus mAoR41

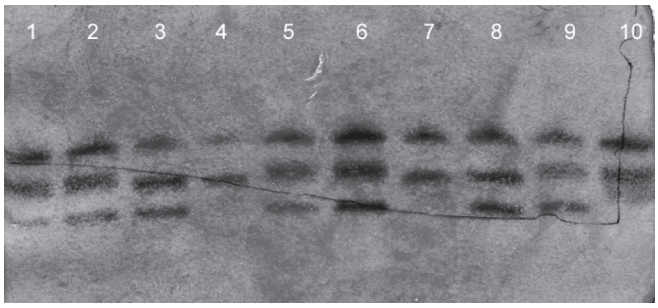

Fig. 1c. Isozyme profile of aspartate aminotransferase (AAT) in 10 accessions

Fig. 1a-c. Sample profiles of markers in 10 accessions

Isozyme analysis with six enzyme systems detected 32 Isozymic bands, of which 28 were polymorphic (87.5%) (Fig. 1c). The number of polymorphic markers varied from 3 (PGM) to 6 (AAT, PER) with a mean of 4.6 polymorphic marker bands per enzyme. The PIC with Isozymes varied from 0.30 (PGI, EST, ACP) to 0.41 (PGM) with an average of 0.33 and MI varied from 1.20 (ACP) to 2.68 (AAT) with an average of 1.51. Of the different Isozyme systems, phosphoglucomutase (PGM), aspartate aminotransferase (AAT) and peroxidase were highly informative with a PIC content of 0.32-0.47 and MI of 1.22 to 2.078. Though

Isozyme markers were limited in number, yet they could distinguish all the accessions.

Each of the marker types employed target different regions of the genome. Of these, SSR corresponds to a specific region of the cashew genome and are found highly reproducible. Though each marker type could distinguish accessions in their own way, the markers of RAPD, SSR and Isozymes were combined to have better differentiation of accessions. By combining, a total of 151 markers were obtained with 123 polymorphic markers (81.5%) with a mean of 5.3 polymorphic markers per primer and had an average PIC of 0.32 and MI of 1.52 (Table 2). Genetic relatedness as revealed by the estimate of Jaccard's coefficient of similarity between the different pairs of accessions varied from 0.39 to 0.77 with a mean of 0.54. High percentage of polymorphism and an average genetic similarity of 54 % suggested high genetic variation and moderate genetic diversity existing among the accessions. The highest genetic similarity (0.77) was observed between VRI-3 of *A. occidentale* and *A. microcarpum* and the lowest genetic similarity (0.39) i.e. high diversity was observed between VRI-3 of *A. occidentale* and *A. pumilum*. Similarly *A. orthonianum* exhibited low similarity with *A. occidentale* and *A. pumilum*. The cluster diagram at 50 % genetic similarity grouped the accessions broadly into three clusters (Fig. 2), The first cluster contained three accessions i.e., *A. pumilum* and its two inter-specific hybrids (V-5 x *A. pumilum* and *A. pumilum* x V-5) and the second cluster contained three accessions *A. orthonianum* and its hybrid *A. orthonianum* x V-5 which in turn paired with Kodippady, an accession of *A. occidentale*. In this group, *A. orthonianum* clustered with *A. orthonianum* x V-5 with a high bootstrap value of 85% and these in turn clustered with Kodippady, a dwarf type of *A. occidentale* with a bootstrap support of only 48%. The third cluster contained predominantly the accessions (purple type, V5 and VRI- 3) of *A. occidentale* and the wild species *A. microcarpum*. In this group, *A. microcarpum* and VRI-3 of *A. occidentale* clustered together with a high bootstrap value of 65 % indicating the close affinity between these two species. Incidentally these two species had shown high genetic similarity. Even morphologically these two species had similar characteristics thereby indicating that *A. microcarpum* is a close relative of *A. occidentale*. Similarly *A. pumilum* and *A. orthonianum* had some resemblance in apple, nut and yield characters but it is the interspecific hybrids which are found robust than their parents in growth but had small fruit and nut characters like their wild relative parent with poor juice quality(sour) (Table 1). The dendrogram made with different groupings were in line with their morphological features observed. The species of cashew with their hybrids were placed in different clusters had a moderate bootstrap support of 49% at their branching/node except for clustering of *A. occidentale* with *A. microcarpum* which had a bootstrap value of 65 %.

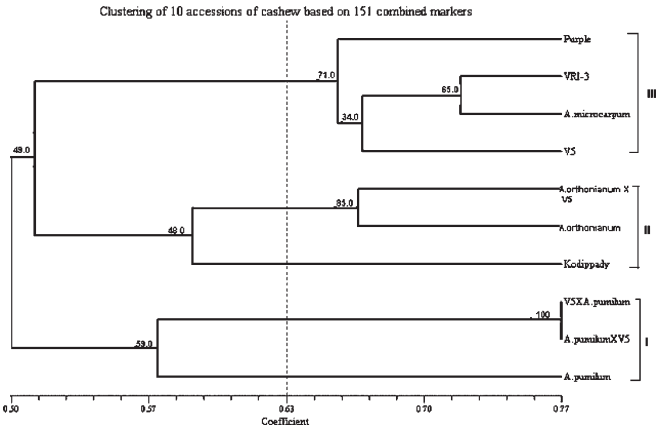

Fig. 2. The UPGMA cluster analysis based on Jaccard's coefficient of genetic similarity. (Figures at the nodes indicate WINBOOT values out of 1000 permutations)

Comparison of markers was done by estimating matrix correlation between the different markers system (Mantel's test), there was low correlation between RAPD and Isozymes and RAPD and SSR. However, a moderate positive correlation (0.58) was observed between Isozymes and SSR makers. This is understandable as the latter are co dominant in nature and RAPD and SSR are altogether different kind of markers. However, the clustering by RAPD analysis and by combined marker analysis was similar. In summary, marker analysis carried out with RAPD, Isozymes and SSR was effective for estimating the genetic diversity and to understand the genetic relationship between the different species and interspecific hybrids of cashew.

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