Comparative Analysis of Morphometric and Molecular Diversity in Cashew (*Anacardium occidentale* L.) Genotypes

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It is noted that seed propagation resorted to, in cashew from the time of its introduction and allogamous nature of the crop have led to the inadvertent spread of non-descript genetic material throughout the state of Goa and the adjoining region, however, with enormous natural variability in it. This cashew seedling progeny in the Konkan region is considered to be the vital source of potent natural recombinants possessing desired commercial traits. Understanding the genetic divergence in cashew was hitherto based on morphological traits and yield components. Keeping in view the comparative analysis of morphometric data with that of molecular data, studies were taken up in 57 cashew genotypes. RAPD analysis differentiated all the cashew genotypes into two broad groups. Grouping of genotypes into six morphometric clusters and two molecular based clusters substantiated the fact that morphometric variables could be always under the influence of environment and thus often would mislead in understanding the actual divergence, where lies, the significance of diversity at molecular level. On the other hand, it would be a great deal of task to actually interpret the molecular data in the total absence of morphometric data. As regards to phenotypic inventory in the background of molecular diversity, the comparative analysis of both molecular results and clustering based on morphometric data reflected rather varied picture about the grouping pattern of genotypes. The studies aptly facilitated the correct selection of parental combination in crop breeding programmes with suitable objectives.

Key Words: Cashew genotypes, Genetic variability, Molecular diversity, Comparative analysis

Introduction

Cashew (*Anacardium occidentale* L.) has gained special status in the international scenario as a plantation crop of considerable foreign exchange earnings. It is cultivated in more than 28 countries of Latin America, Asia, Africa and Australia for its delightful nutritious kernels and apples. This crop was introduced in India on the coasts of Goa in 1570 AD by Portuguese for afforestation and soil conservation. Seed propagation resorted to, till recently, coupled with allogamous nature of the crop has led to the enormous natural variability. The seedling progeny in Goa, thus, is the vital source of potent natural recombinants possessing desired commercial traits, besides having the potential genotypes for specific characters such as resistance or tolerance to biotic/abiotic stress (Mathew and Nagabhushanam, 1988; Desai and Dhandar, 1998). Understanding the genetic divergence in cashew was hitherto based on morphological traits and yield attributes. However, application of molecular markers has emerged as the precise complementary approach to study the genetic diversity (Staub and Serquen, 1996; Lee,1999). Earlier studies indicated the use of DNA markers to reveal the genetic diversity in cashew (Neto *et al.*, 1995; Karihaloo and Archak, 2000; Mneney *et al*., 2001; Dhanaraj *et al.*, 2002; Rout *et al*., 2002; Archak *et al.*, 2003; Samal *et al*., 2003). However, information about the phenotypic inventory in the background of molecular diversity is very meager in cashew. Keeping this in view, importance of interpreting the molecular diversity trends in the light of morphological diversity trends in local cashew genotypes is emphasized in the study.

Material and Methods

Fifty seven genotypes of cashew (Table 1) planted in RBD at 6 m × 6 m spacing in the clonal germplasm bank (of softwood grafts) at ICAR Research Complex for Goa, Old Goa, formed the experimental material for the study. All the trees received the uniform management practices. At the start of this study, cashew trees were of six years age (planted during 1997-98). Observations on 20 morphometric parameters were recorded as per the standard descriptors of cashew (IBPGR, 1986; Swamy *et al*., 1998), consecutively for two seasons 2004-05 and 2005-06).

Morphometric data of 20 growth and yield related attributes was subjected to principal component analysis as suggested by Iezzoni and Pritts (1991). Further, first 14 principal components accounting for 96.5 per cent

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Table 1. Local cashew genotypes and their geographical sources

Sl. no.	Genotype	Accession no.	District	Zone	Village
1	Dhave-1	GCC-94/6	North Goa	Sattari	Dhave
2	Dhave-2	GCC-94/7	North Goa	Sattari	Dhave
3	Dhave-3	GCC-94/8	North Goa	Sattari	Dhave
4	Tiswadi-3	G-CS-4-5	North Goa	Tiswadi	Old Goa
5	Tiswadi-4	G-CS-4-6	North Goa	Tiswadi	Old Goa
6	Tiswadi-5	G-CS-4-7	North Goa	Tiswadi	Old Goa
7	Karapur-1	GCC-94/4	North Goa	Ponda	Karapur
8	Karapur-2	GCC-94/5	North Goa	Ponda	Karapur
9	Ganje-1	GCC-94/1	North Goa	Ponda	Ganjem
10	Ganje-1-2	GCC-94/2	North Goa	Ponda	Ganjem
11	Ganje-2	GCC-94/3	North Goa	Ponda	Ganjem
12	Red Local	GC-CS-10	North Goa	Tiswadi	Old Goa
13	Sweet Round	GC-CS-11	North Goa	Tiswadi	Old Goa
14	Balli-1	GC-94/10	South Goa	Quepem	Balli
15	Goa-1 (Local check)	GC-94/11	South Goa	Quepem	Balli
16	Vengurla-4 (Introduced check)		A high yielding variety introduced from RFRS, Vengurla		
17	Kuddi-1	G-CS-7-12	South Goa	Quepem	Kuddi
18	Kuddi-2	G-CS-7-13	South Goa	Quepem	Kuddi
19	Bardez-6	GC-CS-1-6	North Goa	Bardez	Painowado
20	Bardez-9	G-CS-1-9	North Goa	Bardez	Porvorim
21	Pernem-4	G-CS-10-4	North Goa	Pernem	Torse
22	Zorinth-1	G-CS-6-11	South Goa	Salcette	Sancoale
23	Zorinth-2	G-CS-6-12	South Goa	Salcette	Sancoale
24	Loutolim-1	G-CS-6-2	South Goa	Salcette	Loutolim
25	Loutolim-3	G-CS-6-4	South Goa	Salcette	Loutolim
26	Loutolim-4	G-CS-6-5	South Goa	Salcette	Loutolim
27	Sarvan-1	G-CS-2-1	North Goa	Bicholim	Sarvan
28	SB-2	G-CS-5-3	South Goa	Canacona	Painguinim
29	Tiswadi-2	G-CS-4-4	North Goa	Tiswadi	Ela
30	Tiswadi-7	G-CS-4-9	North Goa	Tiswadi	Old Goa
31	Agonda-1	G-CS-5-6	South Goa	Canacona	Agonda
32	Agonda-2	G-CS-5-7	South Goa	Canacona	Agonda
33	Agonda-5	G-CS-5-12	South Goa	Canacona	Agonda
34	KN 2/98	G-CS-7-7	South Goa	Quepem	Suntegal
35	Sattari-1/00	G-CS-8-8	North Goa	Sattari	Keri
36	Sattari-3/00	G-CS-8-10	North Goa	Sattari	Keri
37	Sattari-4/00	G-CS-8-11	North Goa	Sattari	Keri
38	Sattari Dwarf	G-CS-8-9	North Goa	Sattari	Br. Karmali
39	Sattari-5/00	G-CS-8-12	North Goa	Sattari	Br. Karmali
40	Sattari-30/4	G-CS-30-4	North Goa	Sattari	Br. Karmali
41	Ashley-1	G-CS-6-13	South Goa	Salcette	Raia
42	Silva-1/55	G-CS-	North Goa	Salcette	Varca
43	Valpoi-1	G-CS-8-2	North Goa	Sattari	Valpoi (Dhave)
44	Valpoi-2	G-CS-8-2	North Goa	Sattari	Dhave
45	Valpoi-3	G-CS-8-3	North Goa	Sattari	Polekar Dhave
46	Valpoi-4	G-CS-8-4	North Goa	Sattari	Valpoi (Charvane)
47	Valpoi-5	G-CS-8-5	North Goa	Sattari	Valpoi (Charvane)
48	Valpoi-7	G-CS-8-7	North Goa	Sattari	Valpoi (Charvane)
49	Tudal-1	G-CS-5-15	South Goa	Canacona	Tudal
50	Tudal-3	G-CS-5-17	South Goa	Canacona	Tudal
51	Tudal-4	G-CS-5-24	South Goa	Canacona	Tudal
52	Tudal-5	G-CS-5-14	South Goa	Canacona	Tudal
53	Kholla-2	G-CS-5-9	South Goa	Canacona	Kholla
54	Kholla-3	G-CS-5-20	South Goa	Canacona	kholla
55	Kholker-1	G-CS-7-1	South Goa	Canacona	Cuncolim
56	Mayem-1	G-CS-2-5	North Goa	Bicholim	Mayem
57	Sanguem-1	G-CS-9-3	South Goa	Sanguem	Kirlpal (Dhabal)

variation, were identified and their scores were used for non-hierachical Euclidean cluster analysis. The analysis was done by using SPAR 1 statistical package.

Molecular diversity studies were also carried out for the above 57 cashew genotypes using RAPD marker for its simplicity and cost effectiveness.

Total DNA of each cashew genotype was isolated using tender leaves of new flush by following Cetyl Trimethyl Ammonium Bromide (CTAB) protocol of Murray and Thompson (1980), with minor modifications. Leaf sample (2-3 g) of each genotype ground in liquid nitrogen was extracted in warm CTAB extraction buffer containing 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1% (w/v) PVP and 0.2% (v/v) mercaptoethenol. The pellet of DNA was suspended in T_{10} E_1 for purification by treating with RNase (5µl of 10 mg/ml) followed by deproteinization, using equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The solution was centrifuged at 10,000 rpm for 10 min at room temperature. The aqueous upper layer was collected in a fresh tube and treated with equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer was taken into a fresh tube and 1/10th volume of 3 M sodium acetate and two volumes of chilled absolute ethanol were added and incubated at 4^{o}C for 1 hr. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes, washed with 70 per cent ethanol, air dried and dissolved in 50 µl of T_{10} E_1 buffer for further use. Concentration of DNA was diluted as described in Sambrook and Russel (2001) to working concentration of 100 ng/µl for further use in Polymerase Chain Reaction.

Screening of Primers and PCR Amplification

From seventy four deca-mer RAPD primers (Operon, USA) screened initially using template DNA of cashew genotypes, eventually, a set of 37 primers was selected based on their ability to produce distinct, clearly resolved and reproducible amplicon profiles.

Polymerase chain reactions were carried out in a final volume of 20 µl consisting of 40 ng of Genomic DNA, Assay buffer (1X 10 mM Tris-HCl pH-9.0), 10mM dNTPmix (2.5 mM each), 5pM random primer, 1 U Taq DNA polymerase (Bangalore Genei) in thin walled PCR tubes. Amplification was carried out in Thermal Master Gradient Cycler, (Eppendorf, Germany) with the programme involving a pre-denaturation step at 94.0^{o}C for 3 minutes, 40 cycles each of initial denaturation for 1.0 minute at 94.0^{o}C, primer annealing at 38.0^{o}C for 1.2 minutes and primer extension at 72.0^{o}C for 2.0 minutes with a final extension at 72.0^{o}C for 20 minutes.

The amplified products were resolved by electrophoresis on 1.5 per cent agarose gels stained with ethidium bromide (0.5 µg ml^{-1} of agarose) run at 60 volts in Tris Acetate EDTA buffer (1.0X TAE) for 4 h.

PCR amplicons resolved on agarose gel were scored to generate binary data by giving score 1 and 0 for the presence and absence of amplicon bands, respectively, at different levels of molecular weight, for all the genotypes. Binary data generated for all the primers was subjected to DARwin 5.0.128 programme for interpretation of the results. The dissimilarity matrix of 57 genotypes was generated based on band sharing data and weighted neighbour-joining tree was constructed.

Clustering patterns of genotypes based on morphometric data and molecular data were compared for diversity inventory of cashew genotypes.

Results and Discussion

Principal component analysis based on 20 growth and yield related characters of the selected cashew genotypes revealed that only first 14 principal components qualified, accounting for about 96.52 per cent of the total variation and scores of these 14 principal components grouped all the genotypes into six clusters (Table 2). In cluster-III, a maximum of 15 genotypes grouped together indicating the genetic nearness among them, while 13 genotypes came together in cluster-I. The genotypes having higher nut weight, higher kernel weight and bigger apple with cluster means of 9.52 g, 2.8 g and 102 g, respectively, were grouped together in Cluster-III. In this cluster, the nut weight of the genotypes Tiswadi-4 (No.5), Tiswadi-5 (No.5), Sweet Round (No.13), Kuddi-1 (No.17), Kuddi-2 (No.18), Sattari-5 (No.39), Valpoi-1 (No.43), Valpoi-3 (No.45), Valpoi-5(No.47), Tudal-3 (No.50), Tudal-5 (No.52) and Mayem-1 (No.56) clustered together in cluster-I (Table 2). These genotypes have such traits as small-medium nut size, higher shelling percentage, shorter flowering duration, lesser N-S canopy spread and lower tree height in common. In the same way, the genotypes, Red local, Goa-1, Vengurla-4 and Sattari Dwarf with higher number of shoots per m^2 canopy, number of leaves per twig, number of flowering shoots per square meter canopy, flowering intensity, sex ratio, nuts per panicle, and shelling per cent, but with smaller-medium nut weight and kernel weight came together in cluster-VI, which was completely distinct from the cluster-III. On the contrary, cluster-IV had those genotypes with higher nut weight, apple weight, kernel weight and distinctly higher yielding ones, with higher sex ratio. Tiswadi-3 (No.4), Balli-1 (No.14), Bandez-9 (No.20), Agonda-1 (No.31), KN-2/98 (No.34) and Valpoi-7 (No.48) were the conspicuously distinct genotypes compared to others. The clustering pattern of the genotypes probably reflected the genetic history of the genotypes. This pattern of clustering on

Table 2. Distribution of cashew genotypes in different morphometric clusters and important characteristics

Cluster No.	Total No. genotypes	Genotypes (*Sl.No.)	Main characters and cluster means
I	13	Tiswadi-4 (*5), Tiswadi-5 (6), Sweet Round (13), Kuddi-1 (17), Kuddi-2 (18), Sattari-5 (39), Valpoi-1 (43), Valpoi-3 (45), Valpoi-5 (47), Tudal-3 (50), Tudal-5 (52), Kholker-1 (55), Mayem-1 (56) **(Med. growth and dwarf types)**	Tree height (m) : 3.1 Can. sprd-NS (m): 5.0 Can. sprd-EW(m): 5.2No. shoots/m^2 can:17.7 No. leaves/twig : 13.9 Leaf area/twig (cm^2): 1230 Shelling percent : 30.7
II	9	Dhave-1 (1), Dhave-2 (2), Dhave-3 (3), Karapur-1 (7), Karapur-2 (8), Ganje-1 (9), Ganje-1-2 (10), Ganje-2 (11), Ashley-1 (41) **(Vigourous growth habit)**	Tree height (m) : 5.3 Can. sprd-NS (m) : 6.5 Can. sprd-EW (m): 6.8Leaf area/twig (cm^2): 1809 Nut weight (g) : 7.9Apple weight (g) : 93.0 Nut yield (kg/tree) : 5.4
III	15	Loutolim-3 (25), Loutolim-4 (26), Tiswadi-2 (29), Tiswadi-7 (30), Agonda-2 (32), Agonda-5 (33), Sattari-1 (35), Sattari-3 (36), Sattari-4 (37), Valpoi-2 (44), Valpoi-4 (46), Tudal-1 (49), Tudal-4 (51), Kholla-2 (53), Kholla-3 (54) **(Bold nut and bigger apple)**	Nut weight (g) : 9.52Kernel weight (g): 2.8Apple weight (g) :102
IV	6	Tiswadi-3 (4), Balli-1 (14), Bardez-9 (20), Agonda-1 (31), KN 2/98 (34), Valpoi-7 (48) **(Bold nuts, high yield : yield attributes)**	Sex ratio: 0.33 Nuts/panicle: 2.5 Nut weight (g): 10.6 Kernel weight (g): 3.1 Apple weight (g): 107.0 Nut yield (kg/tree): 7.2
V	10	Bardez-6 (19), Pernem-4 (21), Zorinth-1 (22), Zorinth-2 (23), Loutolim-1 (24), Sarvan-1 (27), SB-2 (28), Sattari-30/4 (40), Silva-1/55 (42), Sanguem-1 (57) **(Bold nut and bigger apple)**	Nut weight (g): 7.9 Apple weight (g): 82.5
VI	4	Red Local (12), Goa-1 (15)), Vengurla-4 (16), Sattari Dwarf (38) **(Yield attributes and yield)**	No. shoots/m^2 can.: 22.0 No. leaves/twig: 16.2 Fl. shoots/m^2 can.: 16.5 Sex ratio: 0.3 Nuts/panicle: 3.9 Shelling percent: 31.9 Nut yield (kg/tree): 6.2

the basis of previous breeding or genetic history, is similar to the report of Swamy *et al.* (2002) and Aliyu and Awopetu (2007) on cashew. Thus, in the present study, the distinct genotypes with potential genetic history for desired traits have been identified, which could be either useful in planning subsequent breeding programmes or could themselves be the evolved potential new varieties for commercial cultivation. In this regard, the short listing of redundant collections and the importance of core collection concept is emphasized by Noirot *et al.* (1996).

Molecular Diversity

The weighted neighbour-joining diversity tree (Fig. 1), constructed based on the RAPD data precisely differentiated all the 57 cashew genotypes in to two broad clusters. Cluster-1, the broad group comprised of 35 genotypes, which in turn indicated eight sub-clusters. While the cluster-II had 22 genotypes with only three sub-clusters.

Phenotypic Inventory in the Background of Molecular Diversity

It is noted that non-hierarchical Euclidean cluster analysis based on morphometric variables grouped all the 57 genotypes into six clusters, while the molecular data based on weighted neighbour-joining diversity tree grouped genotypes into two major clusters. This substantiates the

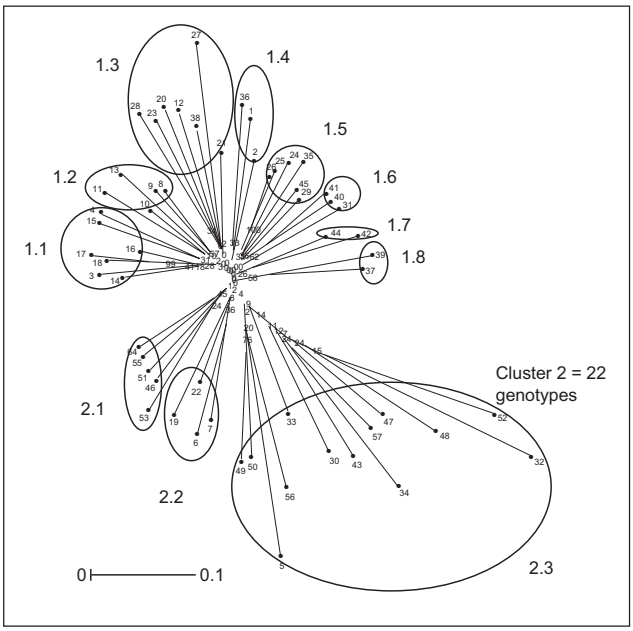

Fig. 1: Weighted neighbour joining tree of 57 cashew genotypes based on RAPD data

fact that morphometric variables are always under the influence of environment and thus often mislead in understanding the actual divergence, where lies, the significance of diversity at molecular level. In general, the morphometric clustering pattern in turn is refined by the molecular diversity pattern, which revealed that actual genetic variability in the germplasm accessions under the study is a population driven mainly by two sources.

Comparison of both diversity approaches, by and large, showed the partial consensus (Table 3) which clearly relates to the necessity of precise understanding of genetic diversity. For instance, the cashew genotypes, *viz.*, Red Local (No. 12), Goa-1 (No. 15), Vengurla-4 (No. 16) and Sattari Dwarf (No. 38) of cluster-VI in morphometric grouping were also separately clustered in major cluster-1 of the molecular diversity tree, however scattered in sub-cluster-1.1 and 1.3 (Table 2). Similarly, the genotypes Tiswadi-5 (No. 6), Tudal 3 (No. 50), Tudal 4 (No. 5), Kholker-1 (No. 55), Mayem-1 (No. 56), Valpoi-1 (No. 43), Valpoi-5 (No. 47) and Tudal-5 (No. 52) of morphometric cluster-I were separately grouped in cluster-2 of weighted neighbour-joining diversity tree (Fig. 1 and Table 2), which were in turn scattered in sub-clusters 2.1 to 2.3. This kind of partial agreement of diversity studies are also reported by Samal *et al.* (2003). It is indeed imperative to precisely understand the genetic make up for the desired traits. In fact, it would be a great deal of task to actually interpret the molecular data in the total absence of morphometric data. Although, classical phenotype features are extremely useful, the efficiency of selection may be reduced by age, developmental stage and by environmental effects on measured traits (Mneny *et al.*, 2001). Any breeding programme will require more reliable information about level of genetic diversity by both approaches for practical significance. In this respect, present studies identified the genotypes with bold nut phenotypic trait scattered across both the major molecular clusters-1 and 2, which indicated that bold nut trait is derived originally from two different genetic back grounds. For instance, the bold nut genotypes such as Tiswadi-3 (S.N0.4), Balli-1 (S.No. 14), Bardez-9 (S.No. 20) and Agonda-1 (S.No.31) and Valpo-2 (S.No.44) are scattered in different sub-clusters of the major molecular cluster-1 derived from one gentic background while bold nut genotypes such as Tudal-1 (S.No.48) and Valpoi-7 (S.No.49), derived from the other genetic background are clustered in sub-cluster-2.3 of the major molecular cluster-2. On the contrary morphometric clustering grouped Valpoi-2 and Tudal-1 in cluster-III and Agonda-1 and Valpoi-7 in cluster-IV although their molecular backgrounds were from two different sources. This information facilitates the breeder in selecting the correct parents truthful to desired traits in crop improvement programmes, which the morphometric diversity studies alone will not provide the breeder with. It is therefore felt that consideration of molecular data supplemented duly by morphological background would be precise practical solution in making the meaningful breeding programmes, since the QTLs (Quantitative Trait Loci) accounted ultimately are through morphological expressions.

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Table 3. Phenotypic inventory in the background of molecular diversity

Weighted neighbour joining tree clusters	Morphometric clusters					
	I	II	III	IV	V	VI
Cluster-1						
Sub-cluster 1.1	*17, 18	3		4, 14,		15, 16
Sub-cluster 1.2	13	8, 9, 10, 11				
Sub-cluster 1.3				20	21, 23, 27, 28	12, 38
Sub-cluster 1.4		1, 2	36			
Sub-cluster 1.5	45		25, 26, 29, 35		24	
Sub-cluster 1.6		41		31	40	
Sub-cluster 1.7			44		42	
Sub-cluster 1.8	39		37			
Cluster-2						
Sub-cluster 2.1	55		46, 51, 53, 54			
Sub-cluster 2.2	6	7			19, 22	
Sub-cluster 2.3	5, 50, 52, 43, 47, 56		30, 32, 33, 49	34, 48	57	

* Serial numbers of genotypes

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