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Molecular characterization and association analysis in cashew using RAPD and ISSR markers

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Abstract

Assessment of genetic diversity in 146 accessions of cashew using RAPD and ISSR markers and its phenotypic evaluation carried out on14 traits revealed considerable variability existing among the accessions. An association analysis was carried out using 96 polymorphic markers (40 RAPD and 56 ISSR) used in diversity analysis with the 14 phenotypic traits studied. Stepwise multiple regression analysis revealed 24 markers in RAPD, 33 markers in ISSR and 48 combined (RAPD and ISSR) markers associating with at least one or more of the phenotypic traits. Several markers associating with a single phenotypic trait and a single marker associating simultaneously with several phenotypic traits (pleiotropy) were observed. The number of markers regressing on each phenotype varied from 1 to 8 in RAPD, 3 to 10 in ISSR and from 3 to 12 with combined markers. Maximum number of RAPD markers (8) were associated with sex ratio followed by 5 markers for flowering intensity (%) and 4 markers for tree spread. Similarly, highest numbers of ISSR markers (10) were associated with inter-node length followed by 8 markers each for percentage of flowering intensity and sex ratio. Highest numbers of combined markers (13) were associated with twig diameter followed by 12 markers for flowering intensity, 11 markers for inter-node length and 10 markers for sex ratio. Least number of markers (1-3) were associated with apple weight followed by number of leaves /twig and kernel weight. R^2 value improved with inclusion of ISSR and combined markers. Markers with significant association are of value if deployed in MAS after due validation.

Keywords: Cashew, genetic diversity, ISSR, marker association, phenotypic trait, RAPD

Introduction

Cashew is an important tropical nut crop grown predominantly in countries like India, Brazil, South Africa and Vietnam. It has been introduced to India in the 15th century and has become naturalized in this part of the subcontinent. Exploitation of cashew has been attempted by both conventional and molecular methods of breeding. The latter is being aided by the use of molecular markers. Many of the economic traits in cashew are quantitative in nature and require systematic crossing work, raising and evaluation of segregating population. As cashew is perennial and heterozygous, development and evaluation of such a population is difficult. An alternative approach to develop regular population is utilization of well-characterized germplasm with adequate phenotype-marker association data in support. This would greatly facilitate marker assisted selection in cashew. Application of markers in the early stage of plant development will improve efficiency of selection and also save considerable time and the cost. So association analysis has been tried in many crops and markers associated with various economic traits are identified. RAPD markers have been associated with quantitative traits in rice (Virk *et al*., 1996) and drought tolerance in tea (Mishra and Sen-Mandi, 2004). Similarly ISSR markers have been associated with leaf and yield attributing traits (Vijayan *et al*., 2006) and biochemical traits in mulberry (Kar *et al*., 2008). RAPD and SSR markers have been associated with mite resistance in coconut (Shalini

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et al., 2007). Roy *et al.* (2006) associated many important agronomic traits using SSR, SAMPL and AFLP markers in bread wheat. SCAR markers were also associated with fiber length in birch (Wang *et al*., 2008). So far, no such attempts have been made in cashew to associate markers with traits. In this paper, association analysis carried out on cashew germplasm using step wise multiple regression analysis is reported.

Materials and methods

Plant material

In this study 146 diverse germplasm accessions maintained at National Cashew Field Gene Bank (NCFGB) of Directorate of Cashew Research, Puttur, India which has both indigenous and exotic collections were used. The germplasm have been maintained in a compact block with 6 plants per accession at a spacing of 6 m x 6 m. They were evaluated after 9 to 10 years after planting and used for collection of leaf samples to extract the DNA.

Phenotypic evaluation

Data on 14 phenotypic traits namely internode length (cm), tree height (m), tree spread (m^2), twig diameter (mm), number of leaves per twig, nut weight (g), sex ratio, weight of apple (g), shell thickness (mm), flowering duration (d), flowering intensity (%), shelling percentage, kernel weight (g) and cumulative yield per plant (g) (6 harvests) were recorded after all the trees have attained maturity stage following IBPGR cashew descriptor.

DNA isolation, PCR amplification and electrophoresis

DNA was isolated from the young leaves of each accession following CTAB buffer extraction procedure with slight modification (Mneney *et al*., 1997). Polymerase chain reaction (PCR) was performed for RAPD and ISSR analysis as described earlier (Thimmappaiah *et al*., 2009). RAPD analysis was carried out with 9 selected Operon primers (OPM-14, OPM-15, OPM-18, OPN-07, OPN-08, OPN-20, OPO-01, OPO-02, and OPO-03) and ISSR analysis was carried out with 10 selected ISSR primers (UBC 834, UBC 856, UBC 855, UBC 857, UBC 825,UBC 827, UBC 841, R11, UBC 865,UBC 873) *i.e.*, sequences of University of British Columbia, Canada. The RAPD and ISSR bands were resolved on 1.5 per cent and 2.0 per cent agarose, respectively and stained with ethidium bromide. The image of the bands was obtained using AlphaImager (USA) gel documentation system.

Data analysis

The mean phenotypic data on 14 phenotypic traits of germplasm were analyzed based on their mean, range, standard deviation, co-efficient of variation, skewness and kurtosis using data analysis of MS Excel 2007. The binary data of markers were scored as '1' for the presence of band and '0' for the absence of band for all the individual markers of RAPD, ISSR and combined markers (RAPD and ISSR).The discriminating power of primers was assessed by calculating percentage of polymorphism, polymorphic information content (PIC) and the marker index (MI). The PIC content of primers was estimated (Powell *et al*., 1996; Smith *et al*., 1997) and the marker index for each was calculated by the formula

PIC= $1-\Sigma fi^2$, where 'fi' is the frequency of i^{th} allele.

Marker Index (MI) = PIC x No. of polymorphic bands.

The binary data (matrix) was used for computing coefficient of genetic similarity (Jaccard, 1908) between all possible pairs of accessions. Cluster analysis was performed using similarity coefficient and unweighted pair group with arithmetic averages (UPGMA) method. A dendrogram was constructed on the basis of Neighbour joining method (Saitou and Nei, 1987) using the software package NTSYS-pc version 2.02 (Rohlf, 1998). Similarly principal coordinate analysis was carried out using the same software.

To carry out an association analysis a total of 96 polymorphic markers consisting of 40 markers in RAPD and 56 markers in ISSR were selected from the diversity analysis and used individually and in combination to regress on 14 phenotypic traits of germplasm following step-wise multiple regression analysis (SMRA) using the statistical package of SPSS version 13.0. The analysis was based on the model:

$Y = a + b_1m_1 + b_2m_2 + b_3m_3 + \ldots b_jm_j \ldots b_nm_n + e$

where, Y = mean of accession for a phenotypic trait (dependent variable) and m_j is a set of independent variables, representing the markers RAPD/ISSR. The b_j represents the partial regression-co-efficient that specify the empirical relationship between Y and m_j, 'e' is the random error of Y that includes environmental variation. This method provided the estimates of relationships between different traits and various markers which were then used to identify the most significant markers of the best fitting multiple regression equation and to test its goodness of fit following Draper and Smith (1981). The square of multiple regression value 'R' denotes R^2. The most appropriate model (multiple regression) was determined by their highest R^2 value. Initially, one variable (marker) models were assessed and the models with highest R^2 value were identified. Then the second variable was added and the best two-variable model was selected based on the same criterion. The model fitting was continued until all the significant variation in Y relative to replicate variation were exhausted. A new variable was added only if the model was significant at 10 per cent probability.

Results and discussion

Molecular markers provide a powerful tool for plant improvement. Among the several DNA markers available RAPD, ISSR, SSR, AFLP, RFLP *etc*. are being used for assessing genetic relationship and as well as for tagging genes with economic traits of interest. Tagging of genes or association of markers with economic traits is very vital for marker-assisted selection. However, this kind of study requires the deployment of mapping population derived from bi-parental crosses such as F_1s, F_1 self, (F_2s), back crossed progeny (BC), F_2 self and back cross self (back cross inbred line-BIL), progenies from F_2 selfing (recombinant inbred lines-RIL) and F_2 back crossed and selfed progeny (near isogenic lines). In a perennial crop like cashew, development of such mapping population is a stupendous task. As there was insufficient mapping population and ready availability of cashew germplasm prompted us to undertake marker-trait association study using germplasm. Germplasm-regression combined marker trait association is the only way in the absence of tight linkage with QTL, non-availability of mapping population and lack of substantial time needed to develop such populations (Ruan, 2010).

Phenotypic evaluation

The mean phenotypic data on 14 phenotypic traits and their descriptive statistics is presented in Table 1. The descriptive data revealed considerable phenotypic variability for different characters. The mean co-efficient of variation varied from a minimum of 16.3 per cent in shelling percentage to 149.3 per cent in sex ratio. In addition to sex ratio, considerable variation was also recorded for number of leaves per twig, cumulative yield per tree, flowering duration, weight of apple and nut weight.

Table 1. Descriptive statistics of 14 phenotypic characters of 146 germplasm accessions

Character	Min.	Max.	Range	Mean	SD	CV (%)	Kurtosis	Skewness
Inter-node length(cm)	0.50	2.50	2.00	1.32	0.396	30.0	-0.379	0.063
Tree height (m)	1.50	8.00	6.50	4.31	1.112	25.8	1.037	0.713
Tree spread (m^2)	1.50	8.40	6.90	5.29	0.932	17.6	2.713	-0.479
Twig diameter(mm)	3.70	10.00	6.30	5.33	0.912	17.1	4.219	1.412
No of leaves $twig^{-1}$	1.00	83.00	82.0	11.08	6.492	58.6	105.248	9.465
Nut weight (g)	2.00	16.78	14.78	6.93	2.534	36.6	1.904	1.157
Sex ratio	0.01	1.39	1.38	0.14	0.209	149.3	22.778	4.728
Weight of apple (g)	10.00	142.8	132.80	60.78	25.95	42.7	0.637	0.836
Shell thickness(mm)	1.50	4.70	3.20	3.14	0.603	19.2	0.368	0.824
Flowering duration (days)	4.20	150.00	108.00	84.07	20.638	24.5	-0.391	0.109
Flowering intensity (%)	14.30	96.80	82.50	57.84	20.048	34.7	-1.026	-0.063
Shelling (%)	15.30	39.50	24.20	27.64	4.508	16.3	-0.253	-0.221
Kernel weight (g)	0.40	4.40	4.00	1.87	0.628	33.6	1.651	0.879
Cum. yield $plant^{-1}$ (kg)	0.40	16.80	16.51	7.49	3.277	43.7	0.150	0.735

Molecular analysis

Genetic diversity in respect of 146 cashew accessions was assessed using RAPD and ISSR markers. Using 9 random primers (RAPD) 46 bands were generated, of which 40 bands were polymorphic (86.9%) with 4.4 polymorphic markers per primer. The number of polymorphic markers varied from 3 (OPM-18) to 6 (OPM-14). The polymorphic information content (PIC) varied from 0.337 (OPN-07) to 0.456 (OPO-01) with an average of 0.403 (Table 2). Similarly the marker index (MI) varied from 1.01 to 2.28 with an average of 1.80. ISSR analysis performed with 10 selected primers generated 61 bands, of which 56 bands were polymorphic (91.8%) with 5.6 polymorphic markers per primer. The number of polymorphic markers varied from 3 (UBC 855) to 7 (UBC 834, UBC 856). The PIC content varied from 0.373 (UBC 825) to 0.470 (UBC 856) with an average of 0.416. Similarly, the marker index varied from 1.13 to 3.29 with an average of 2.35 (Table 3).

To estimate the genetic diversity, the data from RAPD and ISSR were combined which resulted in 107 markers with 96 polymorphic markers (89.7%). The average number of polymorphic bands per primer was 5.1. The high polymorphic value observed with different markers varied from 86.9 to 91.8 per cent indicated the presence of high genetic variation existing among the genotypes. The genetic relationship between the accessions was estimated by computing Jaccard's coefficient of similarity which varied from 0.34 (NRC-22 & NRC-142) to 0.84 (NRC-36 & NRC-231) with an average similarity coefficient of 0.61 which indicated considerable diversity. Using the similarity coefficient values, cluster analysis was performed using neighbour joining method (Saitou and Nei, 1987). The cluster analysis could divide the 146 cashew accessions broadly into two major groups at a genetic distance of 16.0 and one of the major clusters was further divided into two sub-clusters. In this, one of this sub-clusters was further grouped into two smaller sub- clusters indicating in all 4 clusters at a genetic distance of 13.0. The principal coordinate analysis performed was in broad confirmation with the clusters made earlier as depicted in PCO plot (Fig. 1).

Table 2. Polymorphism observed with RAPD primers and markers identified for SMRA with their keys

Sl. No.	Operon/ UBC code	Sequence 5' to 3'	No. off bands	No. of poly-morphic bands	% of polymorphism	*PIC	*MI	Markers selected for SMRA with key no. & bp size	
1	OPM-18	CACCATCCGT	4	3	75	0.394	1.18	1. 650	3. 1500
								2. 750	
2	OPN-08	ACCTCAGCTC	8	5	63	0.368	1.84	4. 500	7. 1200
								5. 675	8. 1450
								6. 975	
3	OPM-15	GACCTACCAC	5	5	100	0.413	2.06	9. 500	12. 1000
								10. 680	13. 1400
								11. 960	
4	OPM-14	AGGGTCGTTC	6	6	100	0.380	2.28	14. 650	17. 1200
								15. 790	18. 1300
								16. 900	19. 1900
5	OPN-07	CAGCCCAGAG	4	3	75	0.337	1.01	20. 650	22. 1500
								21.1200	
6	OPO-02	ACGTAGCGTC	4	4	100	0.429	1.72	23. 800	25. 1100
								24. 850	26. 1200
7	OPN-20	GGTGCTCCGT	5	4	80	0.406	1.62	27. 800	29. 1150
								28. 1100	30. 1200
8	OPO-03	CTGTTGCTAC	6	6	100	0.445	2.67	31. 250	34. 750
								32. 500	35. 900
								33. 700	36. 1200
9	OPO-01	GGCACGTAAG	4	4	100	0.456	1.82	37. 375	39. 1000
								38. 800	40. 1200
		Total / mean	46	40	86.87	0.403	1.80		

* PIC-Polymorphic information content; MI-Marker index

Table 3. Polymorphism observed with ISSR primers and markers identified for SMRA with their keys

Sl. No.	UBC code	Sequence 5′ to 3′	No. of bands	No. of polymorphic bands	% of polymorphism	*PIC	*MI	Markers selected for SMRA with key no. & bp size	
1	834	AGAGAGAGAGAGAGAGYT	7	7	100	0.438	3.07	1. 500	5. 800
								2. 575	6. 950
								3. 650	7. 1000
								4. 700	
2	856	ACACACACACACACACYA	7	7	100	0.471	3.29	8. 575	12. 1000
								9. 700	13. 1025
								10. 800	14. 1250
								11. 900	
3	855	ACACACACACACACACYT	4	3	75	0.378	1.13	15. 600	17. 950
								16. 800	
4	857	ACACACACACACACACYG	6	6	100	0.460	2.76	18. 500	21. 1000
								19. 600	22. 1100
								20. 925	23. 1400
5	841	GAGAGAGAGAGAGAGAYC	6	6	100	0.397	2.38	24. 340	27. 550
								25. 400	28. 725
								26. 500	29. 775
6	873	GACAGACAGACAGACA	6	6	100	0.394	2.37	30. 350	33. 900
								31. 460	34. 1000
								32. 600	35. 2000
7	825	ACACACACACACACACT	7	6	85.86	0.373	2.24	36. 375	39. 650
								37. 425	40. 800
								38. 625	41. 1100
8	865	CCGCCGCCGCCGCCGCCG	6	5	83	0.438	2.19	42. 540	45. 960
								43. 650	46. 975
								44. 700	
9	R-11	GATCATCATCATCATCATCAT CATCATCATC	5	4	80	0.411	1.65	47. 575	49. 1500
								48.1000	50. 2250
10	827	ACACACACACACACACG	7	6	85.86	0.403	2.42	51. 450	54. 1000
								52. 575	55. 1050
								53. 800	56. 1400
		Total/Mean	**61**	**56**	**92**	**0.416**	**2.35**		

*PIC-Polymorphic information content; MI-Marker index

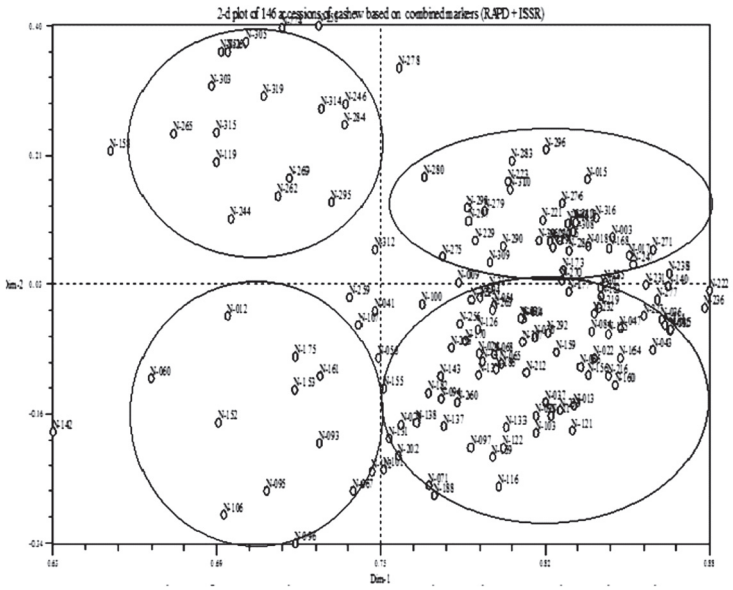

Fig. 1. PCO plot of 146 cashew accessions based on combined markers

Association analysis (SMRA)

The polymorphic markers (96) selected consisting of 40 of RAPD and 56 of ISSR markers used for step-wise multiple regression analysis (SMRA) are listed in Table 2 and 3 respectively. They were used independently and in combination to regress on different phenotypic traits following SMRA. The analysis revealed an association of 22 RAPD, 33 ISSR and 23 combined (RAPD and ISSR) markers with one or more phenotypic traits out of a total of 40, 56 and 96 markers respectively. On the other hand, 18 markers in RAPD and 23 markers in ISSR did not show any association. The number of markers correlated with phenotypic traits varied from 1 (cumulative yield plant^{-1}) to 8 (sex ratio) in RAPD and from 3 to 10 (inter-node length) in ISSR. Similarly with combined markers, association varied from 3 (weight of apple) to 13 (twig diameter). Least number of markers (1-3) was associated with apple weight followed by number of leaves per twig and kernel weight. Among the markers associated, some had positive effect and some negative on the same phenotype (Table 4). The strength of association (%) as indicated by R^2 value increased with addition of markers suggesting involvement of several markers (QTLs) for the same trait. For example, 8 markers in RAPD, 10 markers in ISSR and 12 combined markers showed significant association with sex ratio, inter-node length and flowering intensity (%) with high R^2 values of 34.9, 41.0, 52.2 per cent, respectively. Each of the markers may be associated with QTLs with small effect as described by Santos *et al*. (2011) wherein they identified many QTLs for physico-chemical characteristics of cashew apple. They could observe three markers significantly associated with QTLs identified for oligogenic phenolics, five for total soluble solids, six for total acidity and four for vitamin C content in cashew apple. The range of phenotypic variation (R^2) explained with association markers with different traits varied from 2.9 to 34.9 per cent in RAPD, 8.6 to 41.0 per cent in ISSR and 10.2 to 52.2 per cent in combined markers revealed low to moderate effects of each QTL on phenotypic variation. The percentage of association was found superior with ISSR and combined markers than with only RAPD.

In addition to a number of markers associating with a single trait, a single marker influencing simultaneously on several traits were also observed (pleiotropy). As many as 9 markers in RAPD (OPN-08_{1450}, OPM-15_{960}, OPO-02_{1100}, OPO-02_{1200}, OPN-20_{1100}, OPO-03_{750}, OPO-03_{900}, OPO-01_{800}, and OPO-01_{1200}) were found to regress individually on at least 2 phenotypic traits. Similarly one marker each in RAPD *i.e*. (OPM-14_{790}) was regressing on three phenotypic traits and OPO-03_{500} regressing on as many as 6 phenotypic traits was also observed. In

Table 4. Regression of different markers on phenotypic traits using SMRA with RAPD, ISSR and combined markers (RAPD+ISSR)

Sl no	Phenotype	No. of RAPD markers				No. of ISSR markers				No. of RAPD+ISSR markers			
		+ve assoc	- ve assoc	Total bands	R^2 (%)	+ve assoc	- ve assoc	Total bands	R^2 (%)	+ve assoc	- ve assoc	Total bands	R^2 (%)
1	Internode length (cm)	1	1	2	17.3	7	3	10	41.0	8	3	11	45.8
2	Tree height (m)	1	2	3	15.6	3	0	3	13.0	3	1	4	18.7
3	Tree spread (m^2)	2	2	4	10.9	5	1	6	22.3	6	1	7	24.6
4	Twig diameter (mm)	1	2	3	17.4	1	5	6	23.3	4	9	13	47.5
5	No. of leaves twig^{-1}	2	1	3	10.2	-	-	-	-	1	2	3	10.2
6	Flowering intensity (%)	1	4	5	25.3	3	5	8	39.6	5	7	12	52.2
7	Sex ratio	5	3	8	34.9	2	6	8	35.4	4	6	10	47.3
8	Flowering duration (days)	2	3	5	21.1	2	2	4	19.2	2	3	5	31.2
9	Weight of apple (g)	-	1	1	2.9	2	1	3	18.3	2	1	3	18.3
10	Nut weight (g)	1	2	3	15.5	1	2	3	12.9	2	5	7	23.8
11	Shell thickness (mm)	1	2	3	9.0	3	1	4	15.5	2	3	5	20.1
12	Shelling (%)	2	1	3	12.1	5	2	7	27.7	3	3	6	32.3
13	Kernel weight (g)	1	2	3	17.1	2	1	3	8.6	1	3	4	18.2
14	Cumulative yield plant^{-1} (kg)	-	1	1	3.0	5	2	7	30.5	5	2	7	33.3

R^2 (%) - The percentage variation of a trait explained by the significantly associated markers

ISSR, as many as 12 markers (UBC-834_{650}, UBC-834_{1000}, UBC-855_{600}, UBC-$857_{1000,}$ UBC-841_{725}, UBC-873_{350}, UBC-873_{460}, UBC-825_{650}, UBC-865_{960}, UBC-865_{975}, UBC-827_{575}, and UBC-827_{1050}) were found regressing individually on two phenotypic traits. Similarly one marker UBC-827_{450}regressing on three phenotypic traits, 4 markers (UBC-841_{340}, UBC-873_{900}, UBC-825_{600}, R-11_{575}) on 4 phenotypic traits and one marker (R-11_{2250}) on 5 phenotypic traits were also recorded. Among the combined markers, (R-11_{2250}) was found regressing on as many as 4 phenotypic traits.

As in our studies,Virk *et al*. (1996) identified 12 to 32 RAPD markers associating with quantitative traits in rice and in that they could identify as many as 16 markers associated with culm-length. Mishra and Sen-Mandi (2004) identified a set of RAPD markers associating with drought resistance in tea. Similarly, Shalini *et al*. (2007) identified both RAPD and ISSR markers which could be associated with mite resistance in coconut. Chatterjee and Mohandas (2003) identified an average of 11 ISSR markers associating with 10 yield variables in silkworm out of 147 markers scored. Similarly Kar *et al*. (2008) identified 4 ISSR markers each for protein content and sugar content in mulberry. Earlier in our study we had identified through bulk segregant analysis four RAPD markers associating with nut weight and plant height in cashew (Shobha and Thimmappaiah, 2011). Marker associations as above is attributed to linkage of genes and also for such causes as linkage disequilibrium involving chance association resulting from correlated allele frequencies in small samples (Virk *et al*., 1996). Information on marker association is also useful for selecting putative parents for producing population to map QTLs of a particular trait. This procedure is also useful for identifying the QTLs in the initial stage of screening and exploiting biodiversity in germplasm. From the association established using RAPD and ISSR and in combination it was observed that there were markers which could be tagged with specific traits and could be used in the selection process and in breeding programme (MAS) of cashew. However, these identified markers in this study needs to be validated with the other individuals and population to confirm their association and use them further in marker assisted selection and breeding.

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