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# Fingerprinting of Released Varieties of Cashew based on DNA Markers

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## Abstract

The genetic relationship among 40 varieties of cashew was investigated by analyzing markers derived from ten primers each of RAPD and ISSR and 15 primer sets of cashew SSR. The level of polymorphism in RAPD, ISSR and SSR markers was 71.8%, 87.5% and 93.3% respectively indicated high genetic variation existing among the varieties. Polymorphic information content and marker index computed showed that ISSR and SSR markers as highly informative. The coefficient of genetic similarity (Jaccard) between pair of varieties varied from 0.55-0.96in RAPD, 0.32-0.94 in ISSR and 0.22 to 0.90 in SSR and an average similarity of 0.53, 0.63 and 0.76 respectively revealed predominance of high genetic similarity than diversity. Better genetic differentiation, was achieved by combining markers data. From a total of 196 bands, polymorphism of 81.6% (160 bands) was observed with an average of 4.6 polymorphic bands per primer. The co-efficient of genetic similarity with combined markers varied from 0.46 to 0.86 with an average similarity of 0.66 also indicated narrow genetic distances and low diversity existing between the varieties. Highest genetic similarity (0.86) observed between V-7 and BPP-5 indicated their close relationship and low genetic divergence. On the other hand, lowest similarity of 0.46 observed between Ullal-1 and Jhargram-1 indicated high genetic divergence with these varieties. Both RAPD and ISSR markers detected high genetic similarity (0.95) between the varieties Goa11/6 and VRI-3 and high diversity between Jhargram-1 and Ullal-1 and Jhargram-1 with Chintamani-1. Dendrograms constructed based on each and combined markers identified essentially 10-12 similar groupings except for some minor differences. There was little or no correspondence in the molecular groupings observed between the varieties originated from the same region or similar morphology. The unique markers identified could be used for differentiating the different varieties and for future breeding work in this crop.

### Keywords

Cashew; ISSR; Fingerprinting; Genetic similarity; markers; RAPD; SSR

## Introduction

Cashew (*Anacardium occidentale* L) is an important tree nut crop of India earning a sizeable foreign exchange besides being an employment provider. Though this crop was introduced to India during 16th century, the variability available in terms of gene pool is low. However, the initial efforts made by the different University research centers in the country and by ICAR at CPCRI and then at ICAR-Directorate of Cashew Research (ICAR-DCR) has resulted in a good collection of germplasm and were successful in establishing in various regional Gene banks and National Cashew Field Gene Bank (NCFGB) at ICAR-DCR, Puttur [1]. Considerable efforts made to improve this crop by Breeding through the evaluation of germplasm and hybridization resulted in identification and release of many region specific and national varieties from various research centers and ICAR-DCR. So far, 42 varieties have been released from different centers. Although these varieties could be distinguished based on their morphological descriptors, often, these are misleading as many of these are agronomic in nature and tend to be influenced by the environment in which they grow. Hence, one needs to use reliable markers like DNA markers which are not unduly affected by the environment and are stable in nature. Various DNA markers like RFLP [2], RAPD [3-5], ISSR [6-8], SSR [9-12] and AFLP [13] etc. with their own advantages and disadvantages are available which can be employed for genetic differentiation and identification of various species of plants and animals. The fingerprints generated by these markers could serve as distinguishing features for the varieties and could be used for identification of varieties and for IPR issues. Initially, in cashew, RAPD markers were used for germplasm characterization [14-16] and later Samal et al. [17] used morphological characters and RAPD to distinguish 20 varieties of cashew. RAPD and ISSR markers have been used characterize cashew varieties and selections [18] and germplasm characterization. Even more than one marker types have been employed for genetic differentiation [19-21]. Similarly, we studied genetic variability and genetic relationship existing in 40 released varieties of cashew using PCR markers like RAPD, ISSR and SSR and their results have been presented.

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## Materials and Methods

### Plant materials and DNA extraction

Leaf samples were collected from 40 varieties of cashew (Table 1) from the clonal repository collections available at ICAR-DCR, Puttur, Karnataka, India. Total genomic DNA was isolated from fresh young cashew leaves (1.5 g) collected during the flushing season (winter and early summer) and grinding in liquid nitrogen (-196 °C) following the CTAB extraction buffer method as described by Mneney et al. [22] with slight modification. The extracted DNA was quantified through Hoefer Dyna Quant 200 model of Fluorometer (GE Healthcare, Singapore) and its homogeneity was checked on 0.8% agarose gel electrophoresis.

### RAPD analysis

One hundred and five random primers (Operon Technologies, Germany) were screened with 5 test accessions for polymorphism and reproducibility. From these, 10 decamer primers (OPM-14, OPM-15, OPM-18, OPN-01, OPN-07, OPN-08, OPN-20, OPO-01, OPO-02, and OPO-03) were selected and used for amplification of 40 varieties. PCR amplification was performed in a total volume of 25 µl containing 1 × *Taq* buffer A (10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) (Bangalore Genei, Bangalore), 3 mM $MgCl_2$,

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| Sl.no | Variety | Pedigree | Place of release /state |
|---|---|---|---|
| 1 | Vengurla-1 | Ansur-1 | Maharashtra (MKVVP,Vengurle) |
| 2 | Vengurla-2 | WBDC-VI (Vengurla 37/3) | -do- |
| 3 | Vengurla-3 | Ansur-1×Vetore-56 | -do- |
| 4 | Vengurla-4 | Midnapore Red × Vetore-56 | -do- |
| 5 | Vengurla-5 | Ansur Early × Mysore Kotekar 1/61 | -do- |
| 6 | Vengurla-6 | Vetore-56 × Ansur 1 | -do- |
| 7 | Vengurla-7 | Vengurla-3× M10/4 (VRI-1) | -do- |
| 8 | BPP-1 | T.No.1 × T.No.273 | Andhra Pradesh (ANGRAU) |
| 9 | BPP-2 | T.No.1 × T.No.273 | -do- |
| 10 | BPP-3 | 3/3 Simhachalam | -do- |
| 11 | BPP-4 | 9/8 Epurupalam | -do- |
| 12 | BPP-5 | T.No.1 | -do- |
| 13 | BPP-6 | T.No.56 | -do- |
| 14 | BPP-8 (H2/16) | T.No.1 × T.No.39 | -do- |
| 15 | VRI-1(M10/4) | Vazisodanaipalayam I | Tamil Nadu (TNAU Vridhachalam) |
| 16 | VRI-2 (M 44/3) | T.No.1668 of Kattupalli | -do- |
| 17 | VRI-3 (M26/2) | M 26/2- Edayanchavadi I | -do- |
| 18 | Jhargram-1 | T.No.16 of Bapatla | West Bengal (BCKVV-Jhargram) |
| 19 | Ullal-1 | 8/46 Taliparamba | Karnataka (UAS-Ullal) |
| 20 | Ullal-2 | 3/67 Guntur | -do- |
| 21 | Ullal-3 | 5/37 Manjeri | -do- |
| 22 | Ullal-4 | 2/77 Tuni | -do- |
| 23 | UN-50 | 2/27 Nileswar (T.No.25) | -do- |
| 24 | NRCC Sel-1 (VTH 107/3) | 3/8 Simhachalam | Karnataka (DCR-Puttur) |
| 25 | NRCC Sel-2 (VTH 40/1) | 2/9 Dicherla | -do- |
| 26 | Bhaskara ( Goa11/6) | South Goa | -do- |
| 27 | VTH-174 | Selection from H 4-7 | -do- |
| 28 | S-23 | Local elite type | -do- |
| 29 | VTH 30/4 | Selection from A18/4 | -do- |
| 30 | H 32-4 | Hybrid | -do- |
| 31 | Anakkayam-1 (BLA-139-1) | T.No.139 of Bapatla | Kerala (KAU-Madakkathara) |
| 32 | Madakkathara-1 (BLA-39-4) | T.No.39 of Bapatla | -do- |
| 33 | Madakkathara-2 (NDR-2-1) | Neduvellur | -do- |
| 34 | K-22-1 | Kottarakkara-22 (Layer-23) | -do- |
| 35 | Priyanka (H-1597) | BLA-139-1× 30-1 | -do- |
| 36 | Kanaka (H-1598) | BLA-139-1× H-3-13 | -do- |
| 37 | Dhana (H-1608) | ALGD-1× K-30-1 | -do- |
| 38 | Goa-1 | Balli village (Balli-2) | ICAR Res Centre, Goa |
| 39 | Bhubaneswar-1 | WBDC-V (Vengurla 36/3) | Orissa (OUAT-Bhbaneshwar) |
| 40 | Chintamani-1 | 8/46 Taliparamba | Karnataka (UAS-Chintamani) |

**Table 1:** Details of varieties and elite lines used in the study.

200 µM of each dNTP, 1 µM primer, 1.5 units of *Taq* DNA polymerase (Bangalore Genei) and 50 ng of template DNA. The PCR reaction was carried out in a Mastercycler gradient PCR machine (Eppendorff, Hamburg, Germany) following: an initial denaturation at 95°C for 2 min, cycle denaturation at 94°C for 1min, annealing at 35°C for 1 min, initial extension at 72 °C for 2 min for 35 cycles with final extension at 72°C for 6 min. The amplified products were resolved on 1.5% agarose electrophoresis using 0.5×Tris-acetic acid-EDTA buffer and stained with ethidium bromide (0.5 µg/ml). The image of bands was acquired through UV light using AlphaImager Gel documentation system (Alpha Innotech Corp. USA). The molecular size of the amplicons was determined with reference to the DNA ladder 100 bp (GE Healthcare) and 1 Kb ladder (MBI Fermentas, USA). The PCR reactions were repeated at least twice.

## ISSR analysis

Preliminary screening carried out with 128 ISSR primers (Operon Technologies, Germany) i.e. 100 primers are of UBC primer set #9 (University of British Columbia, Canada) and 28 primers of rice [23], showed satisfactory amplification only in 56 primers. From these amplified primers, 10 primers were selected for their reproducibility and polymorphism and used in the study. Of these, eight were anchored primers namely UBC 834 $(AG)_8YT$, UBC 856 $(AC)_8YA$, UBC 855 $(AC)_8YT$, UBC 857 $(AC)_8YG$, UBC 825 $(AC)_8T$, UBC 827 $(AC)_8G$, UBC 841 $(GA)_8YC$, R11 $G(ATC)_{10}$ and two were non-anchored primers namely UBC 865 $(CCG)_6$ ,UBC 873 $(GACA)_4$. The PCR reactions were performed in the same way as RAPD except for annealing at 55 °C. The amplified products were separated on 2% agarose gel with 0.5×Tris-acetic acid-EDTA buffer by electrophoresis. The gels were stained with ethidium bromide (0.5 µg/ml) and the bands were visualized and acquired under UV light using AlphaImager Gel documentation system. The size of the amplified products was estimated using 100 bp (GE Healthcare) and 1 Kb ladder DNA (MBI, Fermentas, USA).

## SSR analysis

A set of 20 primer pairs of SSR in cashew [24] was got synthesized from Operon Technologies, Germany through M/s Genetix New Delhi. Out of these, 15 primer pairs which were found polymorphic in preliminary study were used. The polymerase reaction mixture (15-20 µl) was prepared in the same manner as described with other markers except for 30 ng template DNA, and1 U of *Taq* polymerase, 1 µM each of forward and reverse primer and the PCR conditions were also similar as above except for the usage of appropriate annealing temperatures which varied with primer sets used. The PCR products were separated on a horizontal 3.5% superfine agarose (GE Healthcare) gel with 50 and 100 kb ladder DNA as reference marker.

## Data analysis

Only distinct and reproducible bands produced by RAPD, ISSR and SSR primers were scored as dominant markers as 'present' (1) or 'absent' (0) with all the varieties screened. 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 [25, 26] 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 × no. of polymorphic bands.

The binary data (matrix) prepared was used for calculating Jaccard's coefficient of genetic similarity between all possible pairs of accessions. Similarity coefficient values estimated were used in

cluster analysis to construct a dendrogram (cluster diagram) using the method of Un-weighted Pair Group with Arithmetic Averages (UPGMA) and Principal Co-ordinate Analysis (PCA) analysis was also carried out following the software package NTSYS-pc version 2.02i [27]. The matrix correlation coefficient (r) was computed using MXCOMP of above package [28] to find relationship between any two dendrograms derived from different data sets (similarity) and their goodness of fit was tested by Z test.

## Results and Discussion

As cashew is perennial and woody in nature, their evaluation is tedious and involves huge cost. Morphological markers are limited and tend to be influenced by environment. Hence, molecular markers can play key role in their characterization and maintenance. Realizing these, PCR based markers like RAPD, ISSR and SSR were employed in this study to characterize 40 varieties of cashew released from different cashew centers.

### RAPD analysis

RAPD analysis with 10 selected primers amplified 75 bands, of which 52 were polymorphic (71.8%). The number of polymorphic bands varied from 2-12 with an average of 5.2 polymorphic bands per primer (Figure 1A). Highest number of bands (12) was produced by OPO 01 primer with all bands polymorphic followed by OPN 11 and OPO 06 The polymorphic information content (PIC) of primers varied from 0.074 to 0.367 with an average of 0.218. Highest PIC was recorded in OPO 01 and OPO 02. The marker index (MI) varied from 0.148 to 4.392 with a mean of 1.263, highest being in OPO 01 followed by OPO 10 and OPO 05 (Table 2). Among the primers, OPO 01, OPO 02 and OPM 18 were highly informative. Unique markers which are variety specific have been identified. For example OPP $10_{1200}$ was unique to BPP 2 variety and OPN $15_{300}$ was unique to BPP 8. Similarly, missing markers (negative markers) were also identified to distinguish varieties. For example, absence of bands in the region of 225bp and 490bp with OPM 20 could identify varieties V-6 and UN 50 respectively. Using combination of primers and their markers, all varieties could be identified (data not given).

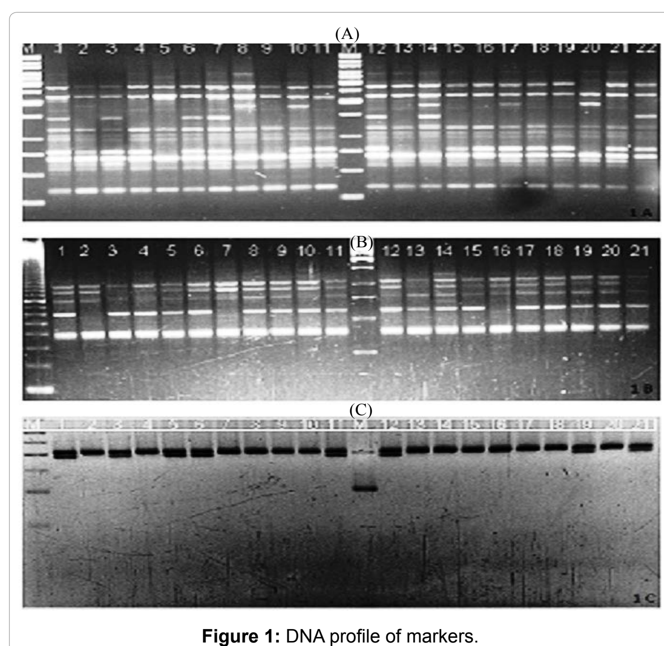

**Figure 1:** DNA profile of markers.

Genetic relationship based on similarity coefficient values varied from 0.55-0.96 with an average similarity of 0.76 indicated high genetic similarity and low diversity existing among the varieties. Highest similarity (0.96) between VRI-3 and Goa 11/6 indicated that these varieties are closely related though they are from two different regions. On the hand lowest similarity (0.55) observed between Jhargram-1 and Ullal- 1 implied that they are highly divergent.

At maximum distance, the cluster analysis with UPGMA method could distinguish 40 varieties broadly in to two major groups in which I- group contained single variety Jhargram-1 and the II group was further separated into two sub groups. The I- subgroup contained single variety Ullal-1 and the II-subgroup could be further divided in to several smaller subgroups (10) with an over total of 10-12 groups. Among the varieties, Jhargram-1 and Ullal-1 were highly divergent and Goa 11/6 and VRI 3 were genetically similar. Earlier, Samal et al. [17] used both morphological characters and RAPD to distinguish 20 varieties of cashew. They reported 86.2% polymorphism from 11 random primers and unique markers for identifying varieties. Among the varieties, high similarity was observed between Ullal-3 and Dhana. Groupings based on RAPD agreed with that of morphological groupings.

### ISSR analysis

In ISSR analysis, with 10 selected primers, a total of 88 bands were generated, of which 77 bands (87.5%) were polymorphic. The number of polymorphic bands varied from 6-12 with an average of 7.7 polymorphic bands per primer (Figure 1B). The highest number of bands (12) was produced by UBC 856 followed by UBC 841, UBC 827 and R-7. The PIC of primers varied from 0.166 to 0.355 with a mean of 0.281 and highest being in UBC 856, UBC 857 and R-7. The MI varied from 1.171 to 4.284 with a mean of 2.229, highest being in UBC 856 followed by UBC 841 and UBC 857 (Table 2). Among the primers UBC 856, UBC 857, UBC 841 and R-7 were highly informative. Higher PIC and MI content of ISSR markers showed that they are more informative than RAPD. ISSR markers detected far more variation than RAPD. As in RAPD, unique markers specific to varieties were identified. For example, UBC $855_{1250}$ was specific to variety V 4 and UBC $827_{500}$ was specific to Ullal 2. Similarly missing markers like 600 and 900 bp with UBC 827 could identify varieties BPP 1 and UN 50 respectively. Combination of primers and their markers could identify all varieties (data not given).

Genetic relationship based on similarity coefficient values varied from 0.32-0.94 with an average similarity of 0.63 indicated predominance of high genetic similarity and less diversity among varieties. As in RAPD, here also highest similarity (0.94) was observed between VRI-3 and Goa 11/6 indicating both these markers were equally effective in genetic differentiation and identification of similarity. The lowest similarity of 0.32 between Jhargram-1 and Chintamani-1 indicated high genetic divergence and low similarity in these varieties.

At maximum distance, the dendrogram based on ISSR markers grouped 40 varieties broadly in to two major groups, in that, I-group contained a single variety Jhargram-1 and the II-group was divided in to two sub groups. The I-subgroup contained a lone variety K 22-1 and the II subgroup was divided further in to several smaller subgroups (10) with an aggregate of 12 clusters from all. Clustering of varieties from same region for example V3 and V4, V1 and V2 in the same group from Vengurle (MH) and Ullal-2 and Ullal-3 from Ullal centers were also observed.

| | Primer code/Locus | Gene sequence (5'-3') | No. of bands | Polymorphic bands | % | *PIC | *MI |
|---|---|---|---|---|---|---|---|
| | | **ISSR** | | | | | |
| 1 | R-7 | CCCGGATCCCACACACACACACACACA | 10 | 8 | 80.0 | 0.321 | 2.568 |
| 2 | UBC 817 | CACACACACACACACAA | 9 | 7 | 77.7 | 0.265 | 1.852 |
| 3 | UBC 825 | GTGTGTGTGTGTGTGTA | 8 | 7 | 87.5 | 0.166 | 1.159 |
| 4 | UBC 827 | GTGTGTGTGTGTGTGTT | 10 | 8 | 80.0 | 0.300 | 2.401 |
| 5 | UBC 834 | TGTGTGTGTGTGTGTGA | 9 | 7 | 77.7 | 0.276 | 1.934 |
| 6 | UBC 841 | GAGAGAGAGAGAGAGAYC | 10 | 9 | 90.0 | 0.318 | 2.859 |
| 7 | UBC 855 | GTGTGTGTGTGTGTGTYA | 6 | 5 | 83.3 | 0.234 | 1.171 |
| 8 | UBC 856 | GTGTGTGTGTGTGTGTYC | 12 | 12 | 100 | 0.355 | 4.264 |
| 9 | UBC 857 | GTGTGTGTGTGTGTGTYG | 8 | 8 | 100 | 0.333 | 2.666 |
| 10 | UBC 865 | CCGCCGCCGCCGCCGCCG | 6 | 6 | 100 | 0.237 | 1.420 |
| 11 | | **Total/Mean** | **88** | **77** | **87.5** | **0.281** | **2.229** |
| | | **RAPD** | | | | | |
| 12 | OPM-15 | GACCTACCAC | 5 | 4 | 80.0 | 0.187 | 0.748 |
| 13 | OPM-20 | AGGTCTTGGG | 7 | 6 | 85.7 | 0.119 | 0.714 |
| 14 | OPO-06 | CCACGGGAAG | 10 | 8 | 80.0 | 0.206 | 1.648 |
| 15 | OPP-10 | TCCCGCCTAC | 8 | 6 | 75.0 | 0.252 | 1.512 |
| 16 | OPN-05 | ACTGAACGCC | 6 | 4 | 66.7 | 0.165 | 0.660 |
| 17 | OPO-03 | CTGTTGCTAC | 8 | 3 | 37.5 | 0.183 | 0.549 |
| 18 | OPN-11 | TCGCCGCAAA | 11 | 2 | 18.2 | 0.074 | 0.148 |
| 19 | OPO-02 | ACGTAGCGTC | 4 | 4 | 100.0 | 0.367 | 1.468 |
| 20 | OPM-18 | CACCATCCGT | 4 | 3 | 75.0 | 0.265 | 0.795 |
| 21 | OPO-01 | GGCACGTAAG | 12 | 12 | 100 | 0.366 | 4.392 |
| 22 | | **Total/Mean** | **75** | **52** | **71.8** | **0.218** | **1.263** |
| | | **SSR** | | | | | |
| 23 | CS-1 mAoR2 | F-GGCCATGGGAAACAACAA R-GGAAGGGCATTATGGGTAAG | 1 | 1 | 100 | 0.278 | 0.278 |
| 24 | CS-2 mAoR3a) | F-CAGAACCGTCACTCCACTCC R-ATCCAGACGAAGAAGCGATG | 2 | 1 | 50 | 0.214 | 0.214 |
| 25 | CS-3 mAoR6c | F-CAAAACTAGCCGGAATCTAGC R-CCCCATCAAACCCTTATGAC | 2 | 2 | 100 | 0.411 | 0.822 |
| 26 | CS-4 mAoR7b | F-AACCTTCACTCCTCTGAAGC R-GTGAATCCAAAGCGTGTG | 2 | 2 | 100 | 0.463 | 0.926 |
| 27 | CS-5 mAoR11c | F-ATCCAACAGCCACAATCCTC R-CTTACAGCCCCAAACTCTCG | 2 | 2 | 100 | 0.209 | 0.418 |
| 28 | CS-7 mAoR12 | F-TCACCAAGATTGTGCTCCTG R-AAACTACGTCCGGTCACACA | 2 | 2 | 100 | 0.293 | 0.586 |
| 29 | CS-8 mAoR16c | F-GGAGAAAGCAGTGGAGTTGC R-CAAGTGAGTCCTCTCACTCTCA | 3 | 3 | 100 | 0.404 | 1.212 |
| 30 | CS-14 mAoR17b | F-GCAATGTGCAGACATGGTC R-GGTTTCGCATGGAAGAAGAG | 3 | 3 | 100 | 0.445 | 1.335 |
| 31 | CS-18 mAoR26 | F-TCCACAAAATCAGCCTCCAC R-GAGCGCTCGTGTCCTGTACT | 4 | 4 | 100 | 0.452 | 1.808 |
| 32 | CS-13 mAoR29c | F-GGAGAAGAAAAGTTAGGTTTGAC R-CGTCTTCTTCCACATGCTTC | 3 | 3 | 100 | 0.348 | 1.044 |
| 33 | CS-15 mAoR33 | F-CATCCTTTTGCCAATTAAAAACA R-CACGTGTATTGTGCTCACTCG | 1 | 1 | 100 | 0.091 | 0.091 |
| 34 | CS-19 mAoR35 | F-CTTTCGTTCCAATGCTCCTC R-TGTGACAGTTCGGCTGTT | 2 | 1 | 50 | 0.259 | 0.259 |
| 35 | CS-20 mAoR41 | F-GCTTAGCCGGCACGATATTA R-AGCTCACCTCGTTTCGTTTC | 3 | 3 | 100 | 0.373 | 1.119 |
| 36 | CS-10 mAoR42c | F-ACTGTCACGTCAATGGCATC R-GCGAAGGGTCAAAGAGCAGTC | 1 | 1 | 100 | 0.133 | 0.133 |
| 37 | CS-17 mAoR44 | F-CACGTTCGCATCATCCAA R-CGTCAGAGATTACGGCATTG | 2 | 2 | 100 | 0.477 | 0.954 |
| 38 | | **Total/Mean** | **33** | **31** | **93.3** | **0.323** | **0.747** |
| 39 | | **Overall Total/mean** | **196** | **160** | **81.6** | **0.281** | **1.318** |

*PIC-Polymorphic information content; MI-Marker index

**Table 2:** Polymorphism observed with different DNA markers in 40 varieties of cashew.

## SSR analysis

STS markers are of choice for fingerprinting due to their high reproducibility, but these markers are rare and recent in cashew. However, SSR markers have been tried in Pistachio [10] and mango [11, 12] for fingerprinting. For SSR analysis in cashew, a set of 15 primers synthesized based on cashew gene sequence [24] was used to generate a total of 33 bands, of which majority i.e. 31 bands (93.3%) were polymorphic (Figure 1C). The number of polymorphic bands (alleles) varied from 1-4 with an average of 2.1 polymorphic bands per primer set (Table 2). Relatively, a low number of bands were amplified in SSR as compared to RAPD and ISSR due to resolution of limited number of bands on superfine agarose but most of the markers produced were highly polymorphic. Highest number of bands (4) was produced by CS 8 primer pair with all the bands being polymorphic followed by CS 20, CS 14 and CS 8. The PIC of primers varied from 0.091 to 0.477 with a mean of 0.323 and highest being in CS 17, CS 4 and CS 18. The MI varied from 0.091 to 1.808 with a mean of 0.747, highest being in CS 18 followed by CS 14, CS 8 and CS 20 (Table 2). SSR markers had high PIC content and low MI than RAPD and ISSR. Genetic relationship based on similarity coefficient varied from 0.22-0.90 with an average similarity of 0.53 indicated a moderate diversity among the varieties.

At maximum distance, the dendrogram based on SSR markers grouped 40 varieties broadly in to two major groups, in that I-group contained a single variety BPP-3 and the II-group was divided in to two sub groups. The I-subgroup contained two varieties namely VRI-2 and VTH 174 and the other (II subgroup) was divided further in to two small subgroups. The I small subgroup contained a lone variety V6 and the II small subgroup was divided into still smaller subgroups (9) with an aggregate of 12 clusters from all. Clustering between BBP 5 and V7 and VRI-3 and Goa 11/6 from different region indicating high similarity and low diversity and clustering of varieties from same region for example like V1 and, V2, V3 and V5 from Vengurle (Maharashtra) center and Ullal-1 and Ullal-4 from Ullal (Karnataka) center were also observed.

Mantel test (1967) made to compare the similarity matrices and dendrograms of all the markers revealed positive low correlations though not significant between RAPD and ISSR (r=0.39) and ISSR and SSR (0.021) indicated weak or poor association between these markers. On the hand, the negative correlations (r=-0.04) between RAPD and SSR indicated absence of association between these markers as they altogether target different gene regions, one being random and another being repetitive gene segment. While positive association between RAPD and ISSR markers may be due to their random nature and the association of ISSR with SSR is due to targeting of repeat sequence in the genome.

## Combined analysis

Though each of the markers was capable of differentiating each of the varieties, the combined data from all the markers were used to give a correct and better estimate of their relationships. Pooling of markers data resulted in 196 bands, of which 160 bands (81.6%) were polymorphic and produced on an average 4.6 polymorphic bands per primer. The PIC of primers varied from 0.074 to 0.477 with a mean of 0.281. The MI varied from 0.091 to 4.392 with a mean of 1.398 (Table 2). Genetic relationship based on similarity coefficient values varied from 0.46-0.86 with an average similarity of 0.66. This indicated high genetic similarity and less diversity existing among the varieties. Highest similarity (0.86) observed between V-7 and BBP1 indicated their close relationship and the lowest similarity of 0.46 observed between Jhargrasm-1 and Ullal-1 indicated low similarity and high genetic divergence.

At maximum distance, the dendrogram (Figure 2) based on combined markers grouped 40 varieties broadly in to two major groups: I-group contained a single variety Kanaka and the II-group was divided further in to two sub groups. The I-subgroup contained a lone variety Jhargram-1 and the II subgroup was divided further in to several smaller subgroups (10) with an aggregate of 12 clusters from all. Clustering of VRI-3 and Goa 11/6 varieties though from different centers showed highest similarity and close relationship with themselves. In contrast, in aggregation of some varieties from same region was also observed. For example BPP 1, BBP 2 and BPP 8 from Bapatla (Andhra Pradesh) were clustered together in same group probably due to their common pedigree as one their parents was T. No. 1 from Andhra Pradesh. Similarly, Ullal 1 and Ullal 4 from Ullal centre (Karnataka) and V1 and V2 from Vengurle (Maharastra) showed clustering together. The principal coordinate analysis (Figure 3) was in confirmation with that of cluster analysis as the two principal coordinates explained 39.5% variation existing among the varieties.

Similarly, in a previous attempt, Archak et al. [18] used combination of RAPD and ISSR markers to fingerprint 35 commercial varieties of cashew. They used in all 94 markers, of which 54 markers were from five RAPD primers and 38 markers were from four ISSR primers. The level of polymorphism with each and combined markers was almost identical (78.0%). The similarity values with combined markers varied from 0.42-0.90 with an average of 0.69 suggesting high similarity and low diversity existing in the varieties has concurrence with our findings. They did not find correlation between molecular data and the pedigree. There were only small differences in the similarity data

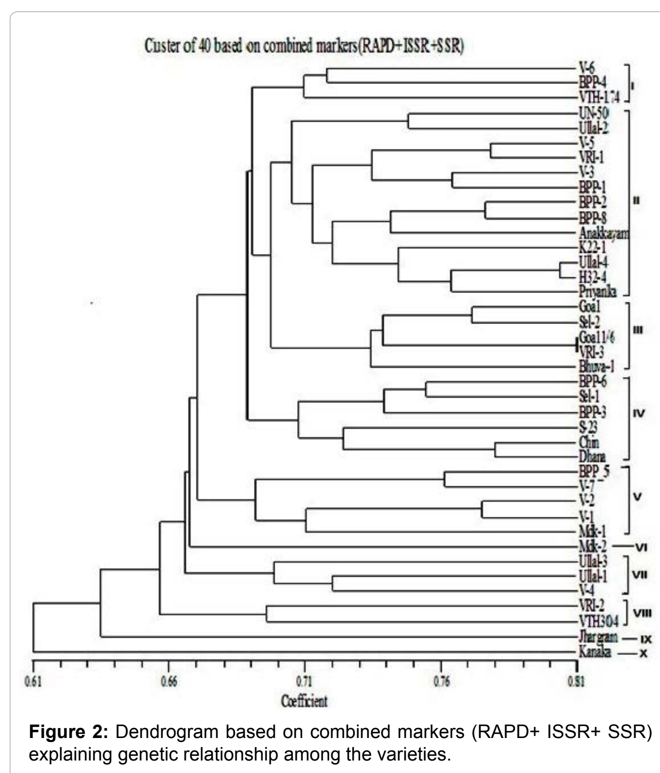


**Figure 2:** Dendrogram based on combined markers (RAPD+ ISSR+ SSR) explaining genetic relationship among the varieties.

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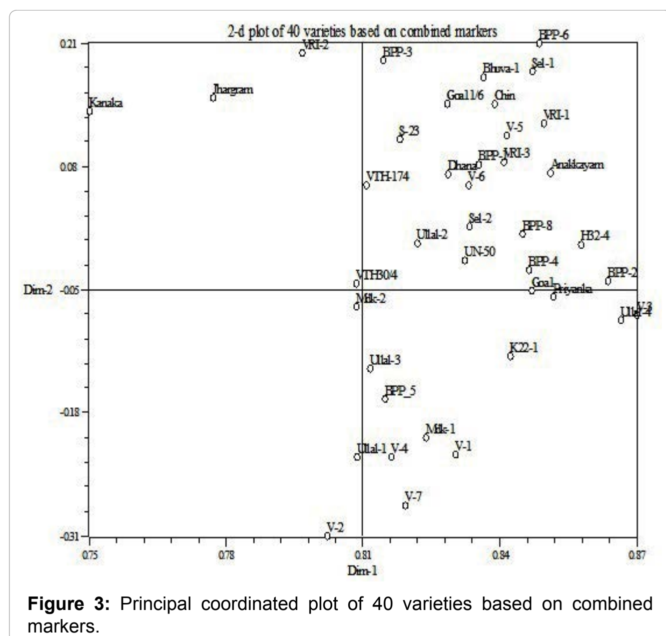


**Figure 3:** Principal coordinated plot of 40 varieties based on combined markers.

between selections and hybrids. As in our studies, more than one marker types have been used in the past to get a better phylogenetic relationship between varieties or accessions. Lopes et al. [20] used RAPD, ISSR and SSR to screen 33 varieties of olive to estimate genetic variability and genetic relatedness. Similarly, Myskov et al. [21] used the same type of markers in rye to assess genetic similarity and genetic relationship and opined that genetic relationship are better illustrated by joining data of more than one marker. Kafkas et al. [19] characterized 69 pistachio accessions using RAPD, ISSR and AFLP and found ISSR better than RAPD and AFLP technique as the best technique.

The study showed that it is possible to differentiate varieties with individual markers as well as by combined markers. However, ISSR and SSR markers were found to be more efficient than RAPD due to their high reproducibility and stringency.

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