Research Article

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# Studies on genetic relationships and diversity in arecanut (*Areca catechu* L.) germplasm utilizing RAPD markers

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## Abstract

In the present investigation, genetic relationship among 60 arecanut germplasm, consisting of both indigenous and exotic accessions, were assessed using 14 polymorphic RAPD primers. The average polymorphism was 6.64 markers per primer. The PIC values among the 14 primers ranged from 0.19 to 0.49. Similarity values among the accessions ranged between 0.68 and 0.93. Cluster analysis revealed two major clusters. The Indian collections *Konkan I*, *Konkan II* and *Maidhan* formed a separate cluster. All the collections from Indonesia, Sri Lanka, Vietnam, Fiji, Solomon Islands, Singapore and China and some Indian collections (*viz*., Andaman and Nicobar Islands and North East germplasm collections) formed a second cluster. The clustering pattern was, in general, in accordance with the geographical origin of the collections. The results obtained from this study is crucial for developing effective management strategies for genetic improvement of arecanut.

*Keywords*: *Areca catechu,* diversity, germplasm, RAPD markers

## Introduction

Arecanut (*Areca catechu* L.) is a monocot belonging to the tribe Arecae and sub-tribe Arecinae in the Arecaceae family. The palm is an unbranched erect, medium-sized, monoecious tree growing in hot and humid tropical regions of the world and its center of origin is considered to be South East Asia (Bavappa *et al.,* 1982). The *Areca* palm, which is highly cross-pollinated, is an allotetraploid, with chromosome number 2n = 32. The genus *Areca* includes 76 species, *A. catechu* being the only cultivated species. Arecanut plays an important role in Asian culture, and is enthrived with economic, religious, cultural and medicinal importance.

Arecanut is commercially cultivated in India, Bangladesh and Sri Lanka. It is also grown in China, Malaysia, Indonesia, Vietnam, Philippines and Thailand, and in several other countries. Arecanut covers an area of 0.72 million hectares with a total production of 0.86 million tonnes (FAO, 2012). The crop provides income and livelihood security to millions of people in the Indian sub-continent, South East Asia and some Pacific islands.

So far, arecanut germplasm has been characterized and evaluated utilizing morphological and yield parameters (Ananda *et al.*, 2000; Rajesh, 2007). However, the genetic diversity information provided by morphological characters is limited and these parameters can be influenced by environmental, genetic and physiological factors. DNA-based molecular markers are an important tool for evaluating levels (Rafalski *et al.*, 1996) and patterns of genetic diversity and have been utilized in a range of plant species and are available in unlimited numbers.

Limited work has been conducted in arecanut for the characterization of germplasm. Ananda and Rajesh (2002) utilized morphological and yield parameters and protein profiles for characterization of arecanut germplasm. Cluster analysis, based on

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protein profiles, did not show any association between geographic and genetic affinities (Rajesh, 2007), highlighting the limitations in using biochemical markers in arecanut. Bagindo (2011) used RAPD for genetic diversity analysis of 30 *Areca catechu* individuals collected in Indonesia (Papua, Sulawesi and Sumatra). Sets of SSR markers were developed by Hu *et al.* (2009) and Zhan *et al.* (2012), while Ren and Tang (2001) optimized protocol for ISSR technique in arecanut, but these markers have not been used for detailed genetic diversity analysis of arecanut germplasm.

No molecular studies have been conducted systematically to estimate the extent of genetic diversity existing among arecanut germplasm accessions. With this perspective, the present investigation, was made to understand the genetic relationships, diversity and geographical correlation among different arecanut accessions, by utilizing RAPD markers.

## Materials and methods

### Plant materials

Sixty different arecanut accessions, representing different geographical regions, were selected for the study (Table 1). The accessions include 43 collections from India (comprising collections from Konkan I, Konkan II, Maidhan, North East, Andaman and Nicobar Islands) and 17 germplasm collections from South East Asia and Pacific region (from Sri Lanka, China, Vietnam, Indonesia, Singapore, Fiji and British Solomon Islands). These accessions conserved and maintained in the field gene bank at ICAR-CPCRI (RS), Vittal, Karnataka, India which is considered to be the largest assemblage of arecanut germplasm collection in the world (Ananda and Sane, 1999).

### Genomic DNA extraction

From each accession, leaf samples were collected from four different palms representing the accession. Tender and soft textured leaf samples (1g) were cut into small pieces and macerated into fine powder using liquid nitrogen. DNA was extracted by using the standardized protocol (Rajesh *et al.*, 2007). The DNA purity intactness was checked in 0.8 per cent agarose gel stained with ethidium bromide following the protocol of Sambrook *et al.* (1989). Genomic DNA was quantified using the spectrophotometer, and finally the samples were diluted to a concentration of 10 ng $\mu L^{-1}$. After extraction of genomic DNA, equal quantities of DNA from four samples representing, each accession, were pooled together and used for RAPD analysis.

### RAPD analysis

Initially, six representative accessions from diverse geographic origins were screened with 60 random decamer primers of the series OPM, OPAF and OPC (M/s Operon, USA) to identify polymorphic primers. RAPD analysis was carried out using the standardized protocol (Rajesh *et al.*, 2007). Amplification reactions were carried out in 15 µL volume containing 20 ng genomic DNA as template, 200 µM of each dNTPs, 15 pmol of random primer, 1X *Taq* DNA buffer and 0.5 U of *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd., India). PCR was performed in DNA Engine thermal cycler (BIO RAD) with an initial denaturation at 94 °C for 5 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 42 °C for one minute and extension at 72 °C for 1 minute, finally ending with one cycle of 72 °C for 10 minutes. After amplification, the PCR product was stored at -20 °C till electrophoresis. Ampified products were mixed with 3µL of 6X gel loading dye (0.25 per cent bromophenol blue and 40 per cent sucrose in $H_2O$) before loading. The amplification products were subjected to electrophoresis and resolved in a 1.5 per cent agarose gel using 1X TBE buffer at 90 V for 2 h in electrophoresis unit. The ethidium bromide stained gels were visualized and photographed using a gel documentation and analysis system (Bio-Rad, USA). Amplification reactions were repeated thrice for checking reproducibility.

### Data scoring and analysis

Scoring of RAPD bands was carried out by considering only the clear, unambiguous and reproducible bands. Markers were scored for the presence and absence of the corresponding band among the different genotypes. The scores '1' and '0' were given for the presence and absence of bands, respectively. DNA band size was estimated by comparing the DNA bands with a 1 Kb DNA ladder as reference (Bangalore Genei Pvt. Ltd., India). The binary data matrices data of 60 different

**Table 1. List of 60 arecanut accessions used in the current study and their Country/State of collection**

| Sl.No. | Accession |
|---|---|
| | **A. Exotic collections** |
| 1. | Fiji-I (Fiji) |
| 2. | Mangala (China) |
| 3. | Srilanka-I (Srilanka) |
| 4. | Indonesia-IV (Indonesia) |
| 5. | Indonesia-VI (Indonesia) |
| 6. | Saigon-I (Vietnam) |
| 7. | Saigon-II (Vietnam) |
| 8. | Saigon-III (Vietnam) |
| 9. | Srilanka-II (Srilanka) |
| 10. | Singapore (Singapore) |
| 11. | Br.Sol.Islands-I (British Solomon Islands) |
| 12. | Br.Sol.Islands-II (British Solomon Islands) |
| 13. | Br.Sol.Islands-III (British Solomon Islands) |
| 14. | Fiji-II (Fiji) |
| 15. | Saigon-V (Vietnam) |
| 16. | Saigon-VI (Vietnam) |
| 17. | Saigon-VII (Vietnam) |
| | **B. Indian collections** |
| | Andaman and Nicobar Islands (ANDNIC) |
| 18. | Andaman & Nicobar Islands-I |
| 19. | Andaman & Nicobar Islands -II |
| 20. | Andaman & Nicobar Islands -III |
| 21. | Andaman & Nicobar Islands -IV |
| 22. | Andaman & Nicobar Islands -V |
| 23. | Andaman & Nicobar Islands -VI |
| | *Konkan -I (Maharashtra)* |
| 24. | Asud |
| 25. | Varand |
| 26. | Murud |
| 27. | Diveagar-I |
| 28. | Diveagar-II |
| 29. | Shriwardhana-B |
| 30. | Shriwardhana-M |
| 31. | Karle |
| 32. | M.Raigad |
| | *Maidhan (Karnataka)* |
| 33. | Sagar |
| 34. | Hirehalli Tall (H. Tall) |
| 35. | Hirehalli Dwarf (H. Dwarf) |
| 36. | S.K. Local |
| | *Maidhan (Kerala* |
| 37. | Wayanad |
| | *Konkan -II (Goa)* |
| 38. | Velling Gauthan |
| 39. | Curti |
| 40. | Ponda-SR |
| 41. | Khandolla |
| 42. | Tamsule |
| 43. | Banstari |
| 44. | Keri- B |
| | *Konkan II (Maharashtra)* |
| 45. | Talkatta-1 |
| 46. | Talkatta-2 |
| 47. | Vengurla |
| 48. | Daboli |
| 49. | Ratnagari |
| | North East (Assam) |
| 50. | Badarpur-I |
| 51. | Badarpur-II |
| 52. | Badarpur-III |
| 53. | Hylakandi |
| 54. | Cachhar |
| | North East (Meghalaya) |
| 55. | Dauki Hills-1 |
| 56. | Dauki Hills-II |
| 57. | Mowlong-I |
| 58. | Mowlong-II |
| 59. | Jawai-I |
| 60. | Jawai-II |

accessions were entered into the NTSYS pc package (Exeter Software, USA) (Rohlf, 1993). The data were analyzed to generate Jaccard's similarity coefficient. Similarity coefficients were used to construct a dendrogram using UPGMA (Unweighted Pair Group Method with Arithmetic Average) and SAHN (Sequential hierarchical and nested clustering) routine. The same binary data for 60 accessions was also subjected to principal component analysis (PCA) with NTSYS software package version 2.02 and the 3-D score plot was generated.

The average Polymorphic Information Content (PIC) was calculated by applying the formula given

by Powell *et al.* (1996) and Smith *et al.* (1997) PIC = $1 - \Sigma fi^2$, where i = 1 to n and $f_i$ is the frequency of the $i^{th}$ allele. The number of alleles refers to the number of scored bands. The frequency of an allele was obtained by dividing the number of accessions where it was found, by the total number of accessions. The PIC value provides an estimate of the discriminating power of a marker. Nei's gene diversity (Nei, 1973), Shannon's information index (Lewontin, 1972) were also calculated.

## Results and discussions

### Degree of polymorphism

Initial screening of a few representative arecanut accessions, from diverse geographic origins with 60 RAPD primers, revealed only 14 primers to be polymorphic and these selected 14 primers were used to assess genetic relationships of the 60 accessions (Table 2). Annealing temperature was enhanced to 42 °C and each of the experiments was repeated thrice to ensure reproducibility of the RAPD results. Only bands that were consistently and unambiguously scorable were identified and scored. A total of 93 reproducible polymorphic and 27 monomorphic bands were identified and scored as RAPD markers. Depending on primers, 5 to 13 reproducible bands were detected in the 100 to 2500 bp size range. A maximum of 13 bands were produced using the primer OPAF-6 primers, while only five amplified fragments were obtained with OPAF-19 primer. Gel profile of arecanut accessions using the primer OPAF-2 is given in Fig. 1.

The PIC values ranged from 0.19 (OPAF 2) to 0.49 (OPAF 16), Shannon's information index values ranged between 0.53 (OPAF 19) to 2.35 (OPAF 6) and gene diversity ($h_i$) ranged between 0.24 (OPAF 6) to 0.39 (OPM 13). The percentage of similarity varied from 0.68 and 0.93 considering the 60 accessions together. Maximum similarity was observed between Shriwardhana-M and Shriwardhana-B (0.93) and the minimum similarity was observed between Diveagar-I and Saigon-I (0.68).

### Cluster analysis

The UPGMA-derived dendrogram is shown in Figure 2. The dendrogram revealed two major clusters, which appear to be equally divergent, at 68 per cent similarity level.

**Table 2. Details of random primers, number of polymorphic and monomorphic bands, percentage polymorphism, Polymorphic Information Content (PIC), Shannon's Index, Gene diversity ($h_i$) and amplified product size**

| Sl. No. | Primer name | Nucleotide sequence from 5' to 3' | Total no. of bands | No. of polymorphic bands | No. of monomorphic bands | % of poly-morphism | Amplified fragment size (bp) | PIC value | Shannon's Index | Gene diversity |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | OPM2 | ACAACGCCTC | 10 | 8 | 2 | 80 | 300-1450 | 0.47 | 2.04 | 0.31 |
| 2. | OPM7 | CCGTGACTCA | 9 | 7 | 2 | 78 | 300-2000 | 0.42 | 1.67 | 0.36 |
| 3. | OPC1 | TTCGAGCCAG | 8 | 6 | 2 | 75 | 300-1500 | 0.22 | 0.86 | 0.37 |
| 4. | OPC7 | GTCCCGACGA | 9 | 7 | 2 | 78 | 300-1500 | 0.41 | 1.48 | 0.25 |
| 5. | OPM5 | GGGAACGTGT | 6 | 5 | 1 | 83 | 300-1400 | 0.36 | 0.97 | 0.30 |
| 6. | OPM12 | GGGACGTTGG | 7 | 5 | 2 | 71 | 350-1400 | 0.38 | 1.04 | 0.33 |
| 7. | OPM13 | GGTGGTCAAG | 10 | 8 | 2 | 80 | 400-1500 | 0.23 | 1.28 | 0.39 |
| 8. | OPM18 | CACCATCCGT | 7 | 5 | 2 | 71 | 500-2000 | 0.40 | 1.40 | 0.33 |
| 9. | OPAF2 | CAGCCGAGAA | 12 | 10 | 2 | 83 | 300-2500 | 0.19 | 1.13 | 0.34 |
| 10. | OPAF6 | CCGCAGTCTG | 13 | 9 | 4 | 69 | 250-2000 | 0.44 | 2.35 | 0.24 |
| 11. | OPAF12 | GACGCAGCTT | 8 | 7 | 1 | 88 | 400-1500 | 0.47 | 1.85 | 0.39 |
| 12. | OPAF16 | TCCCGGTGAC | 8 | 6 | 2 | 75 | 100-1500 | 0.49 | 1.75 | 0.27 |
| 13. | OPAF10 | GGTTGGAGAC | 8 | 7 | 1 | 88 | 400-1500 | 0.28 | 0.56 | 0.28 |
| 14. | OPAF19 | GGACAAGCAG | 5 | 3 | 2 | 60 | 500-1500 | 0.29 | 0.53 | 0.30 |
| | Total | | 120 | 93 | 27 | 78 | | | | |
| | Mean | | 8.57 | 6.64 | 1.93 | | | | | |

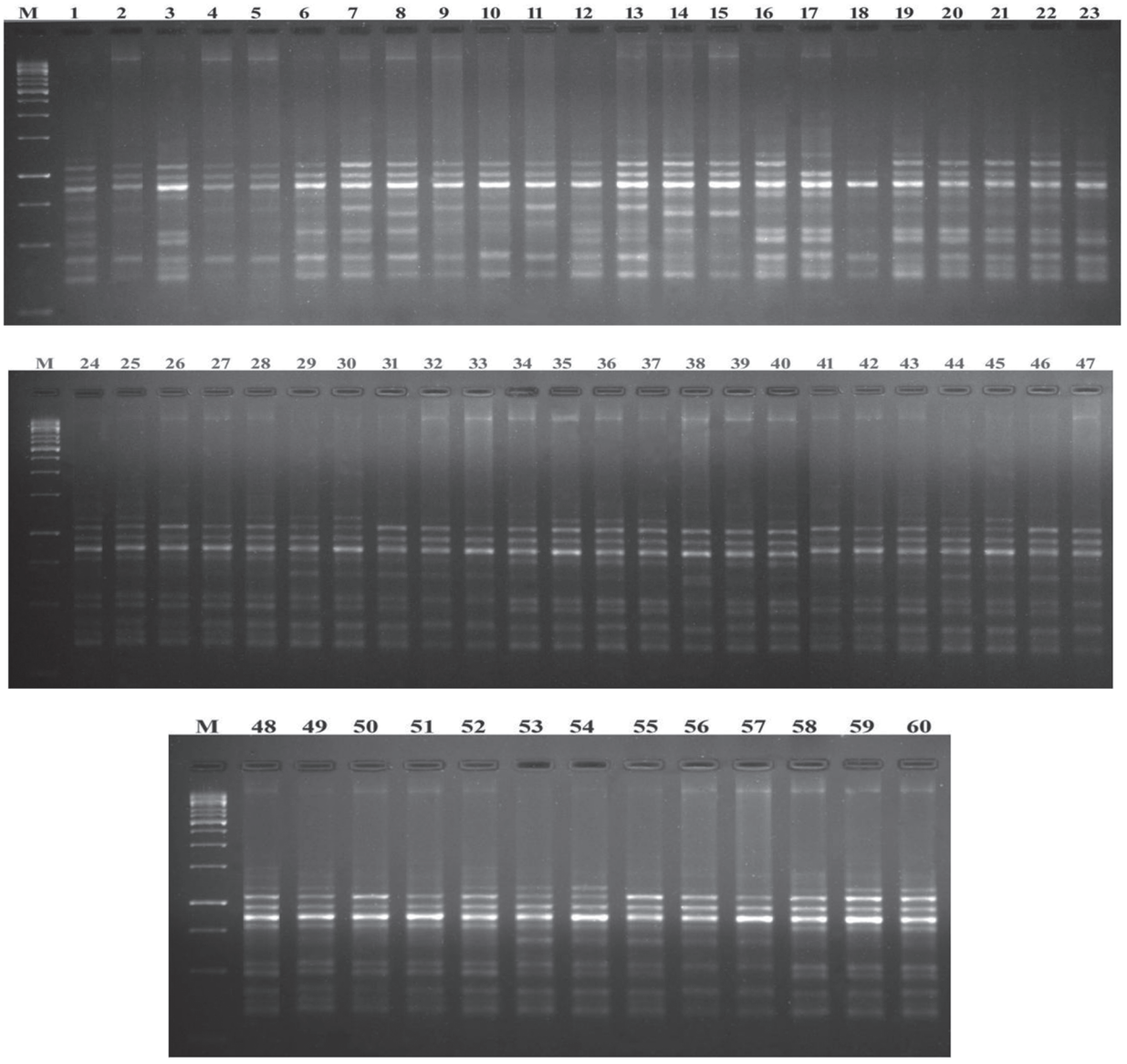


**Fig. 1. RAPD banding profiles generated using primer OPAF-2 in the 60 arecanut accessions. Standard 1 Kb ladder (M) was used as a reference.**

Cluster I consisted of two sub-clusters at 71 per cent similarity level. All exotic accessions, along with Indian accessions from North East and Andaman and Nicobar Islands, were clustered together in a major cluster. It is noteworthy that two accessions, namely S.K. local and Wayanad, shared similarity with arecanut accessions from North East and Andaman and Nicobar islands and the exotic ones. Close relationship among accessions from North East India was observed and the genetic polymorphism among them was found to be narrow. The cluster pattern displayed grouping of accessions from North East India, exotic accessions and those from Andaman and Nicobar Islands, which corresponded to their geographical proximities (Fig. 3).

Cluster II was comprised exclusively of the Indian accessions, *viz*., *Konkan I, Konkan II* and *Maidhan* collections. Here too, two major sub-clusters were split at around 70 per cent similarity levels. The indigenous accessions *viz*., *Konkan I*, *Konkan II* and *Maidhan*, showed average diversity

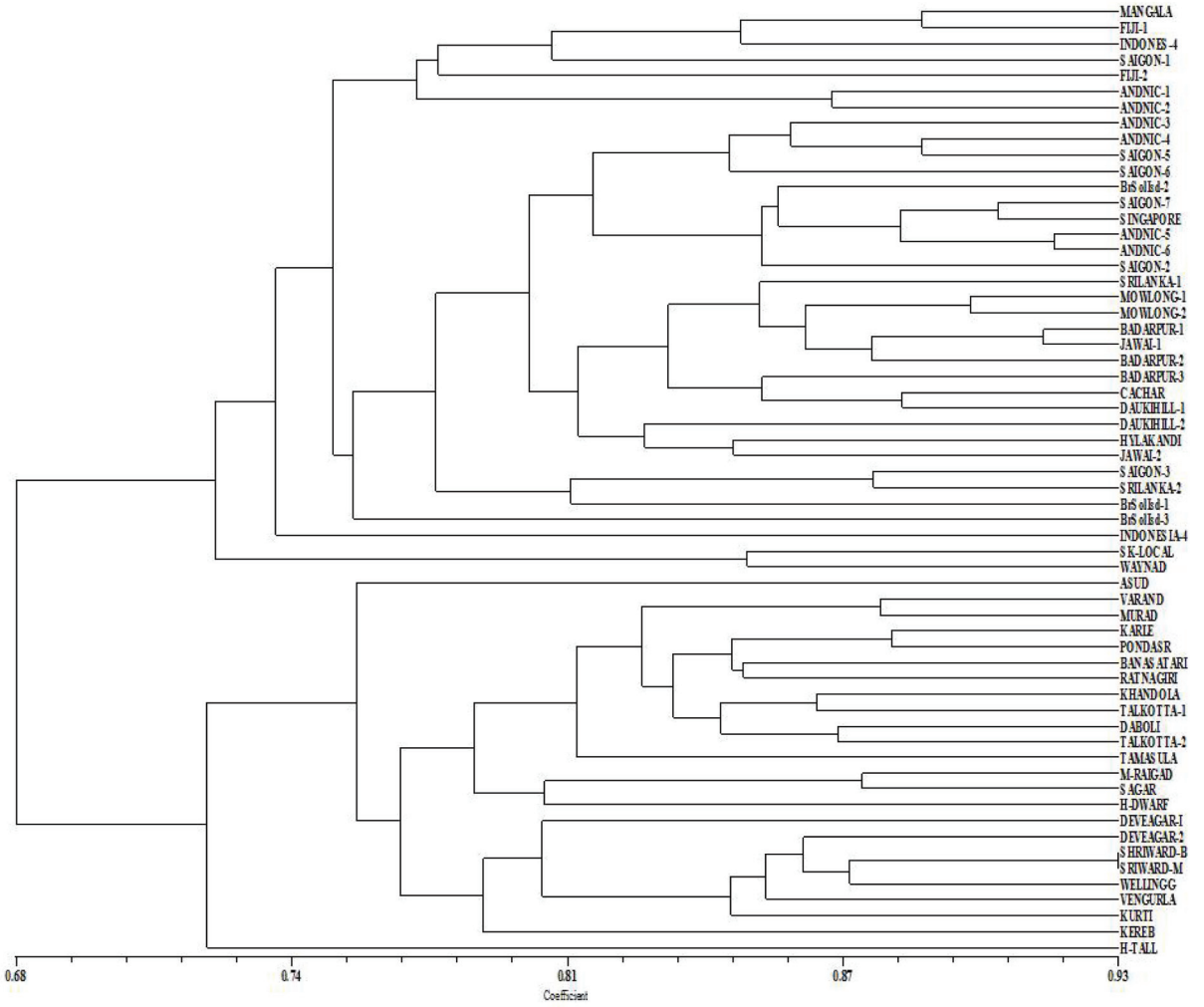


**Fig. 2. A UPGMA dendrogram based on RAPD data for the 60 arecanut accessions.**

amongst themselves and were highly divergent from accessions from North East India, non-Indian and Andaman and Nicobar Islands, as revealed by the clustering patterns. Hirehalli Tall formed a distinct accession in this group.

Arecanut is one of the important plantation crops of India having economic, religious, cultural and medicinal importance. The largest collection of arecanut germplasm in the world is maintained in the field gene bank at ICAR-CPCRI. Despite the outstanding agronomic and socio-economic significance of arecanut palm, attempts to improve knowledge about the biodiversity of arecanut germplasm have been limited to the phenotypic description of cultivars. The critical evaluation of these germplasm, for estimating the extent of genetic diversity present in them, is necessary for their utilization in future arecanut breeding programmes and also, identification of accessions, especially the duplicates. Palms like coconut, oil palm, palmyrah and date palm have been subjected to many intensive studies for their variability, based on morphological as well as molecular markers, but such detailed studies have not been undertaken in arecanut till date. However, a deeper insight of the genetic diversity of arecanut warrants an urgent priority to guide the use of this diversity in crop improvement programmes. The results of such a study could definitely lead to formulation of better strategies for future crop improvement programmes in arecanut.

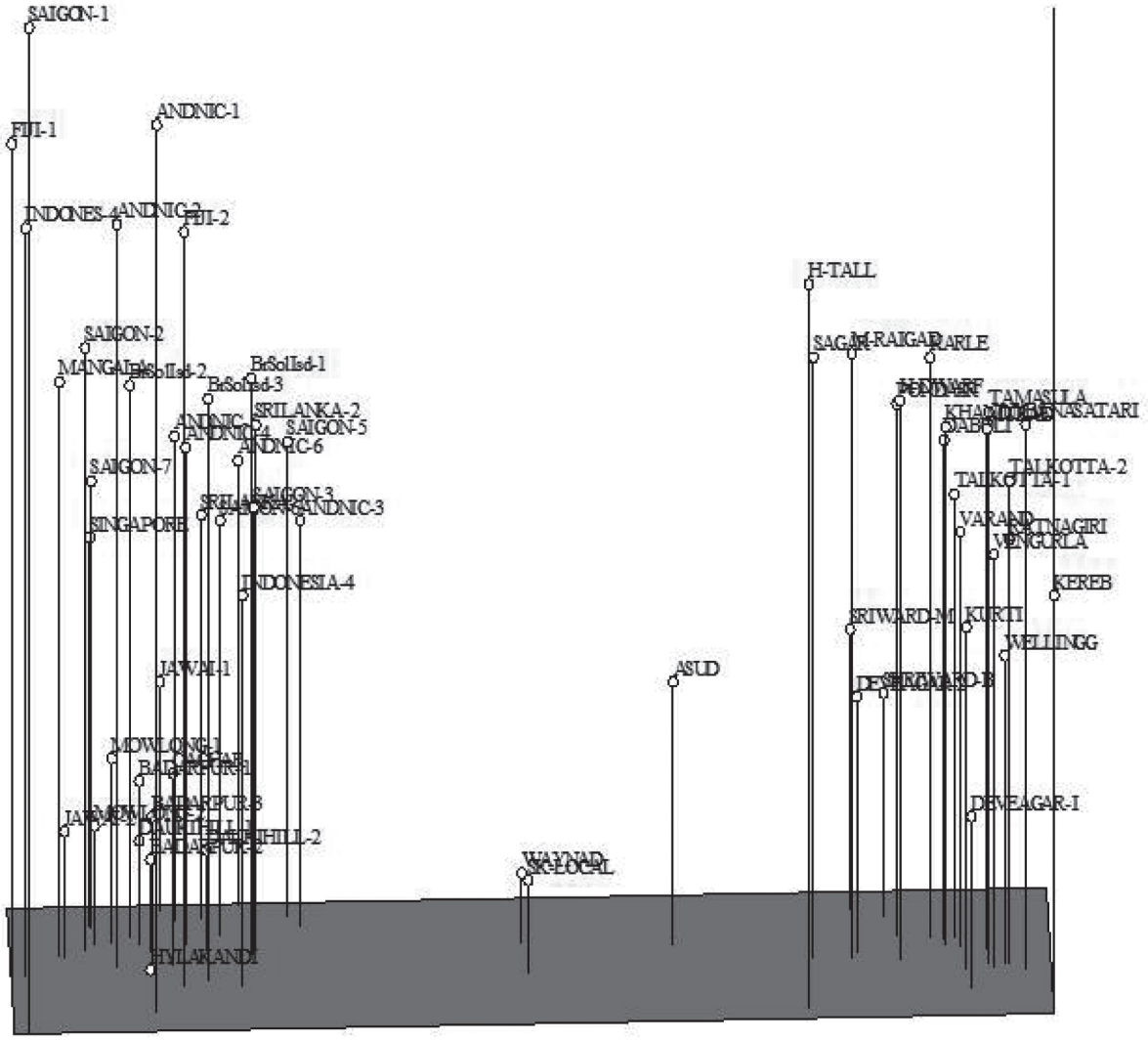


**Fig. 3. A PCA 3-D plot based on RAPD data for 60 arecanut accessions.**

With the above perspective, the present investigation was undertaken to analyze the extent of genetic diversity present in arecanut germplasm from different parts of the world, using RAPD markers. Although RAPD markers do suffer from reproducibility issues (Sedra *et al.*, 1998; Trifi *et al.*, 2000), mainly due to mismatch annealing (Neale and Harry, 1994; Demeke *et al.,* 1997), the results of the present investigation in arecanut virtually discriminated and characterized samples according to their geographic origins indicating the suitability of this marker system in analyzing arecanut germplasm accessions. For higher reproducibility, the annealing temperature was enhanced to 42 °C and the experiments were repeated thrice for validation of the banding patterns.

The percentage of polymorphic bands among the arecanut accessions (78%) indicates the existence of a high level of genetic diversity among the accessions analyzed. The level of polymorphism, reflected by the number of polymorphic bands per primer (6.64), was also high which corroborates the earlier observation of existence of higher variability amongst arecanut germplasm accessions (Ananda and Rajesh, 2002) and also, the suitability of RAPD technique for germplasm characterization studies in arecanut, in which genome information is lacking.

Indigenous accessions from North East India and Andaman and Nicobar Islands were closely grouped with exotic accessions, which could point to a common genetic origin of arecanut in the South Eastern Asian region, and its subsequent movement and secondary diversification in the Indian peninsula *via* Andaman and Nicobar Islands and North East India, as proposed by Zumbroich (2008). The dendrogram deduced from the RAPD marker analysis appear to correlate with previous speculation regarding the origin of crop (Bavappa *et al.,* 1982) and also provides evidence on the geographical distribution of the species from the centre of origin. Unlike the findings of Rajesh (2007), such relationship between geographical distribution and genetic affinity suggests that RAPD are more suitable molecular markers than protein profiles. A study of distant accessions identified through cluster analysis, indicated the existence of least similarity between Diveagar-I (an accession from Konkan region, India) and Saigon-I (an accession from Vietnam). These two accessions could be used for hybridization studies in order to obtain highly heterotic progenies.

The results of the study thus provides the first basic information on the genetic relationship amongst arecanut accessions from diverse geographical regions and can form the basis of advanced studies on germplasm characterization and conservation, breeding programs and selection of possible parents to generate mapping populations of arecanut accessions.

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