

Genetic Relationship between Cultivars of Areca Nut (*Areca catechu* L.) Determined by RAPD

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ABSTRACT

In the present studies, genetic relationships among eleven cultivars of Areca nut (*Areca catechu* L.) obtained from Western Ghats regions of India were analysed using RAPD markers. PCR-amplifiable DNA was isolated using the CTAB method and 146 amplified fragments were obtained using 19 random primers. The genetic dissimilarity matrix, which was calculated based on Squared Euclidian Distances, revealed a maximum genetic distance of 47% between cultivars, 'Mohit Nagar Interse' and 'Mohit Nagar' and the minimum genetic distance (22%) was between the genotypes 'Maidan Local' and 'Sree Mangala'. The Ward's method of cluster analysis grouped all the individuals on a dendrogram into two major clusters 'A' and 'B' at 29 linkage distances with two sub-clusters in cluster 'A'. The sub-cluster 'A₁' consisted of nine cultivars in two minor clusters 'A_{1a}' and 'A_{1b}' linked at 27 distances. The sub-cluster 'A₂' consisted of one cultivar 'Sumangala' linked with 'A₁' at 28 linkage distance. The cluster 'B' consisted of one cultivar 'Mohit Nagar'. The present study showed moderate genetic diversity among the cultivars of Areca nut. The RAPD analysis proved to be a quick, simple and significant testing method to assess genetic diversity among Areca nut populations studied.

Keywords: cluster analysis, genetic diversity, RAPD-PCR, STATISTICA

INTRODUCTION

Areca nut (*Areca catechu* L.) is the most profitable plantation crop grown in humid tropics of India, realizing highest economic returns per unit area. Apart from tobacco, alcohol and caffeine, it is the most commonly used substance and globally, an estimated 600 million people are believed to indulge in the habit (Raja *et al.* 2007). Areca nut is usually consumed alone, in its natural form, as a processed product, or as a key ingredient of the chewing quid (Warnakulasuriya 2002). The exact origin of areca nut palm is not fully known as there are no fossil records of the genus *Areca*. Geologically the palm extends to the Cretaceous period of upper Mesozoic era (Mahabale 1982). It is a native of Cochin (China), the Malaya Peninsula and neighbouring islands (Watt 1889). The exact native country of areca nut is uncertain (de Condolle 1886) but the range of distribution in a locality where the plants are thought to be wild may throw some light on its origin (Furtado 1933). The history of areca nut chewing was established among the Aryans at least 2000 years ago (Gode 1961).

Areca nut plays a prominent role in the religious, social, cultural and economic life of Indians. The economic product is the fruit called "betel nut" and is used mainly for masticatory purposes. In India, areca nut is used as an offering during religious ceremonies (Murthy 1968). It is cultivated in the plains and foot hills of Western Ghats and North Eastern regions of India. Area and production in different states show that Karnataka, Kerala and Assam account for over 90% (Rajagopal 2004). It is vital to small land holders as a source of sustainable income. It is grown over an area of 2,64,000 ha with an annual production of 313,000 t (Chowdappa *et al.* 2003). It is estimated that nearly ten million people depend on the Areca nut industry for their livelihood in India. The quality, variety and types

of Areca nut vary from one place to another.

According to Bentham and Hooker, the genus *Areca* is the first one in the family *Palmae*, in their treatise *Species Plantarum*. The genus expanded rapidly from its monospecific status and it is believed to compose of about 76 species. Among these, *A. catechu* is the only cultivated species and nuts of a few other species such as *A. triandra* Roxb. and *A. concinna* Thw. are also used as a masticatory (Murthy and Pillai 1982). Chewing areca nut is the most common mode of administration although its use as a masticatory has been on the decline over the passed few decades, with the incursion of modern ways of living among rural populations, particularly in South East Asia (Rajagopal 2004). Recent clinical diagnostic studies have revealed that chronic areca nut chewing has been strongly linked to the development of oral submucous fibrosis (Murti *et al.* 1995; Warnakulasuriya *et al.* 1997; Gupta *et al.* 1998), a chronic progressive pre-cancerous condition characterised by fibrosis of the mucosal lining of the oral cavity and occasionally the upper digestive tract (Maher *et al.* 1991). The development of cancer in the upper aero-digestive tract is increased markedly in patients with consumption of areca nut (Warnakulasuriya 2002).

Diversity of areca nut germplasm based on morphological characteristics has been performed. Beccari (1919) recognised four cultivars of areca nut and termed them as varieties *silvatica*, *batanensis*, *longicarpa* and *communis* based on the size and shape of fruit and kernel. Based on their height areca palm are classified into tall, semi-tall and dwarf types. The height ranges between 60 and 360 cm as recorded at the seventh year of age (Rajagopal 2004). Murthy and Bavappa (1962) identified 64 cultivars based on fruit size, from Kerala, Karnataka and Maharashtra. Based on the variation in stomatal characters, concerning number of stomata per unit area four cultivars were identified by Ba-

vappa (1966). Bavappa and Pillai (1976) found highly significant differences in respect of number of leaves shed, spadices and female flowers produced, nut set, number of nuts harvested and, weight and size of nuts among thirteen cultivars of *A. catechu* from eight countries. Cultivars available in Malaya, Sri Lanka and South India have been designated by local names (Aiyer 1966; Nambier 1954).

Identification of cultivar and estimation of genetic diversity using phenotypic markers have several limitations, especially in perennial crops. Molecular differences, which can be detected using DNA and protein-based markers, are more authentic and unaffected by environmental factors (Dhanraj *et al.* 2002). Hence, the characterisation of genotypes at the genetic level provides the first step towards more efficient conservation, maintenance and utilisation of existing genetic diversity (Prakash *et al.* 2002). Among DNA-based molecular markers, RAPDs (Randomly Amplified Polymorphic DNA) proved as excellent tool to estimate genetic diversity and relationships among genotypes (Williams *et al.* 1990). They are simple, versatile, relatively inexpensive, and can detect slight genetic differences and help in identifying duplicates in the populations. RAPD markers also have been used successfully to study genetic diversity and relatedness among perennial crops by our research group such as mango (Hemanth Kumar *et al.* 2001), guava (Prakash *et al.* 2002), cashew (Dhanraj *et al.* 2002) and jackfruit (Simon *et al.* 2007). To the best of our knowledge no research work on genetic studies has been carried out on areca nut cultivars despite its importance. In the present study, RAPD markers were used to estimate genetic diversity and assess relationships among 11 cultivars of areca nut.

MATERIALS AND METHODS

Plant materials

The plant material used for the study comprises 11 cultivars of areca nut collected from Western Ghats and maintained at the University of Agricultural Sciences, Shimoga, India. The cultivars were 'Maidan Local' (MDL), 'Sumangala' (SUM), 'Mangala' (MAG), 'Mohit Nagar Interse' (MNI), 'Sirsi Local' (SSL), 'Mohit Nagar' (MHN), 'Sree Mangala' (SMG), 'Mangala Interse' (MGI), 'Thirhalli Local' (TTL), 'Saigon-12' (SG12) and 'Sagar Local' (SGL). About 50 g of young and healthy leaves that were free from pest and disease damage was harvested individually from the field, wiped with 70% ethanol, air-dried and stored at 4°C in sealed polythene covers until the isolation of DNA.

DNA isolation and purification

All the reagents and chemicals were obtained from Bangalore Genei, Bangalore, India of molecular biology grade. DNA was extracted from the stored leaf of areca nut by the modified cetyl trimethyl ammonium bromide (CTAB) protocol as described by Simon *et al.* (2007). Specifically, 500 mg of leaf was powdered using liquid nitrogen and was mixed with 10 ml extraction buffer, preheated to 65°C, containing 100 mM Tris-base, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 3% (w/v) CTAB, 2% polyvinyl pyrrolidone and 1% β-mercaptoethanol, then incubated at 65°C for 30 min with gentle shaking. The mixture was cooled to room temperature, to which 10 ml cold 24:1 (v/v) chloroform:isoamylalcohol was added and the contents were mixed well. After centrifugation at 6,000 × g for 20 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform:isoamylalcohol step was repeated until a clear supernatant was obtained. To the supernatant 5 M NaCl was added (0.5 v/v) and mixed gently followed by addition of 0.8 vol of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C, and then centrifuged at 8,000 × g for 20 min. The resulting pellet was washed with 70% (v/v) ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Two μg RNase (Bovine pancreatic ribonuclease) was added to each sample which was incubated for 3 h at 37°C, mixed with an equal volume of phenol and centrifuged at 6,000 × g for 20 min. at room temperature. This step was

Table 1 Genetic dissimilarity matrix of 11 areca nut cultivars based on polymorphism of RAPD markers.

MNI	0																			
SGL	30	0																		
SUM	33	35	0																	
MGI	41	41	28	0																
SSL	32	26	35	29	0															
SG12	27	29	32	32	25	0														
TTL	41	33	32	26	23	36	0													
MDL	42	30	37	33	24	35	23	0												
SMG	46	34	39	31	26	39	27	22	0											
MHN	47	39	42	32	35	36	32	29	31	0										
MAG	50	41	41	37	30	45	29	34	22	29	0									

followed by a washing with an equal volume of 1:1 (v/v) phenol:chloroform and then with chloroform alone. DNA was precipitated overnight at 4°C with 0.5 vol of 5 M NaCl and 1 vol of cold isopropanol and the resulting pellet obtained after centrifugation was dissolved in TE buffer, analysed on an agarose gel and quantified using a spectrophotometer (ND-8000, NanoDrop Technologies, Wilmington, USA).

PCR amplification

The PCR amplification protocol followed was according to Williams *et al.* (1990) with minor modifications. Of the 38 primers screened using the bulk DNA, 19 showing prominent bands were selected for RAPD-PCR analysis (Table 1). Reproducibility of the primers was tested by repeating the PCR amplification three times under similar conditions. PCR reactions were carried out in a volume of 25 μl containing 25 ng template DNA, 150 μM each dNTP, 1.5 mM MgCl₂, 1.5 unit *Taq* DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India), 5 pmol primer (OPA, OPB, OPC, OPD, OPE, OPF, OPG, OPH, OPI, OPJ and OPK series, Operon Technologies, Alameda, CA, US) in PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100). Amplifications were performed in a Corbett Research Thermocycler (Corbett Research Mortlake, New South Wales, Australia), programmed for an initial denaturation at 94°C for 4 min., followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, primer extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were resolved in a 1.2% (w/v) agarose gel, visualised and documented using an Alpha Digidoc system (Alpha Innotech, San Leandro, CA, US).

RAPD profile analysis

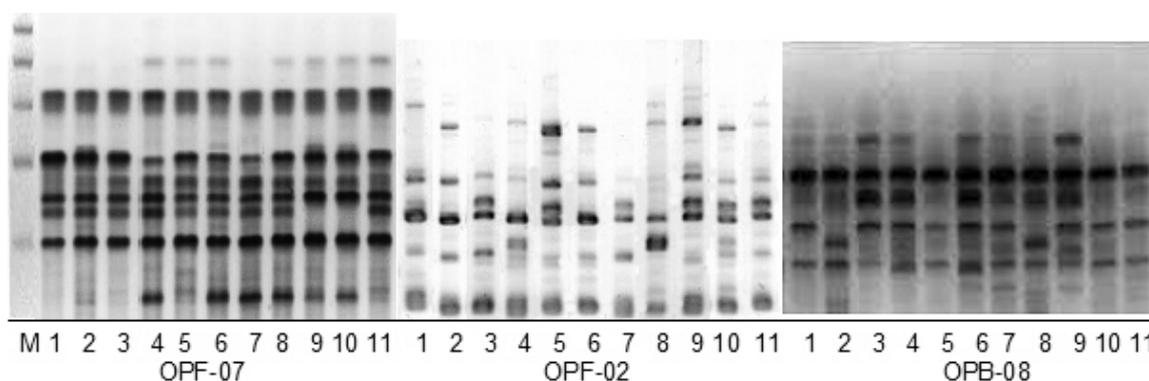
Amplified fragments from each primer was manually scored for their presence (1) or absence (0) and a matrix of the different RAPD phenotypes of all eleven primers was assembled for statistical analysis by STATISTICA computer package (STATISTICA for Windows, StatSoft Inc, Tulsa, OK, USA, 1996). The sizes of the fragments were estimated using 500 bp standard DNA markers (Bangalore Genei, Bangalore, India), co-electrophoresed with the PCR products. A genetic dissimilarity matrix was developed using Squared Euclidean Distances, which estimates all pair-wise differences in the amplification products (Sokal and Sneath 1973) and a cluster analysis was based on Wards method using a minimum variance algorithm (Ward 1963). Principal Component Analysis (PCA) was used to make a multivariate statistical analysis of the RAPD data (Sokal and Sneath 1973).

RESULTS AND DISCUSSION

Genetic diversity among cultivated plant species is important for the efficient utilization of plant genetic resources. Understanding the spatial organization of genetic diversity within and among plant populations is of critical importance for the development of strategies designed to preserve genetic variation (Hamrick 1983; Brown and Briggs 1991). It has been shown that species with limited gene flow, i.e. with restricted seed and/or pollen movement, have consider-

Table 2 RAPD-PCR primers. The sequence and level of polymorphism of selected polymorphic primers in areca nut.

S. №	Primer	Sequence (5' to 3')	Total № of bands	№ of polymorphic shared bands	№ of polymorphic unique bands	№ of monomorphic bands
1	OPA-02	TGCCGAGCTG	6	2	0	4
2	OPA-04	AATCGGGCTG	9	5	1	3
3	OPA-11	CAATCGCCGT	6	2	1	3
4	OPA-13	CAGCACCCAC	11	6	2	3
5	OPA-15	TTCCGAACCC	6	4	0	2
6	OPA-16	AGCCAGCGAA	2	0	0	2
7	OPB-01	GTTTCGCTCC	8	2	1	5
8	OPB-08	GTCCACACGG	9	7	0	2
9	OPB-12	CCTTGACGCA	6	2	0	4
10	OPB-18	CCACAGCAGT	6	4	0	2
11	OPD-05	TGAGCGGACA	9	6	1	2
12	OPD-13	GGGGTGACGA	7	3	2	2
13	OPD-20	ACCCGGTCAC	12	9	1	2
14	OPF-02	GAGGATCCCT	15	13	2	0
15	OPF-03	CCTGATCACC	6	4	0	2
16	OPF-06	GGGAATTCCGG	7	3	1	3
17	OPF-07	CCGATATCCC	9	5	0	4
18	OPF-08	GGGATATCGG	4	3	1	0
19	OPF-10	CCTCTAGACC	8	4	0	4
Total			146	84	13	49

**Fig. 1** Gel profile of areca nut obtained using OPF-07, OPF-02 and OPB-08 primers. Lanes 1-11 contain the amplification profile obtained using the cultivars (MDL, SUM, MAG, MNI, SSL, MHN, SMG, MGI, TTL, SG12 and SGL). Lane M contains 500 bp standard DNA markers.

ably more among-population variation for total amount of genetic diversity (Schoen and Brown 1991). Evidently, RAPD technology is a rapid and sensitive technique, which estimates relationships between closely and more distantly related species.

Genetic studies are rare or lacking in areca nut because they have not been the subject of much scientific investigation. However, morphological and cytological studies have been performed in *A. catechu* (Bavappa 1963) and a breeding programme based on cytogenetics has been conducted (Bavappa and Nair 1982). Cultivar improvement for better yield of areca nut has been performed based on morphological markers (Bavappa and Ramachander 1967). However, the use of morphological or physiological characters is strongly influenced by environmental factors (Shulman *et al.* 1984). Varieties in pomegranate are often classified based on characters such as number of leaves, spadix and flower, height of the plant, size and shape of fruits and seeds. Ananda and Anuradha (1999) reported that in areca nut in India, variability exists with respect to vegetative and nut components among the indigenous accessions. Despite many local varieties, very few are commercially utilised (Aksoy 1995; Llacer *et al.* 1995).

PCR amplification followed a standard protocol (Williams *et al.* 1990) with minor modifications, producing good amplifications with 25 ng of template DNA. The amplifications using 1 unit of *Taq* DNA polymerase and 1.5 mM MgCl₂ produced intense and clear banding patterns. The primer-screening step resulted in 19 decamer primers which detected good polymorphisms (Table 2). The amplification profiles of total genomic DNA from eleven areca

nut accessions with 19 random primers produced a total of 146 fragments ranging in size from 250 bp to 2.5 kbp with an average of 7.7 bands per primer. Of the 146 bands, 84 (57.5%) were polymorphic and shared between at least two individuals, 49 (33.6%) were monomorphic and common to all the individuals. Thirteen (8.9%) were polymorphic and unique. The number of fragments produced by a primer ranged from 2 (OPA 16) to 15 (OPF 02). The pattern of RAPD fragments produced by the random primer OPF-02, OPF-07 and OPB-08 are shown in Fig. 1. The dissimilarity matrix obtained using Euclidian Distance (Sokal and Sneath 1973) is shown in Table 1. The highest genetic dissimilarity (47%) was between cvs. 'Mohit Nagar Interse' and 'Mohit Nagar', while the least genetic dissimilarity (22%) was noticed between cvs. 'Maidan Local' and 'Sree Mangala'.

In the dendrogram (Fig. 2), all 11 cultivars were divided into two major clusters 'A' and 'B'. Cluster 'A' consisted of 10 cultivars that segregated into two sub-clusters 'A₁' and 'A₂' with nine and one cultivars, respectively. Two unique bands produced by primers OPA-13 and OPA-15 were specific to all the cultivars of cluster 'A'. The sub-cluster 'A₁' was divided into two minor clusters 'A_{1a}' and 'A_{1b}' at 27 linkage distance. The minor cluster 'A_{1a}' consisted of one cultivar 'Mohit Nagar Interse'. The minor cluster 'A_{1b}' consisted of eight cultivars segregated into three groups at 26 linkage distance. The group I of 'A_{1b}' comprised of one genotype 'Sagar Local'. Cvs. 'Maidan Local', 'Sree Mangala' and 'Mangala' were closely linked at 22 linkage distance in group II and were closely linked to cvs. 'Thirthalli Local' and 'Sirisi Local' at 23 linkage distances. The genotype 'Saigon -12' stood as a separate entity in the group II.

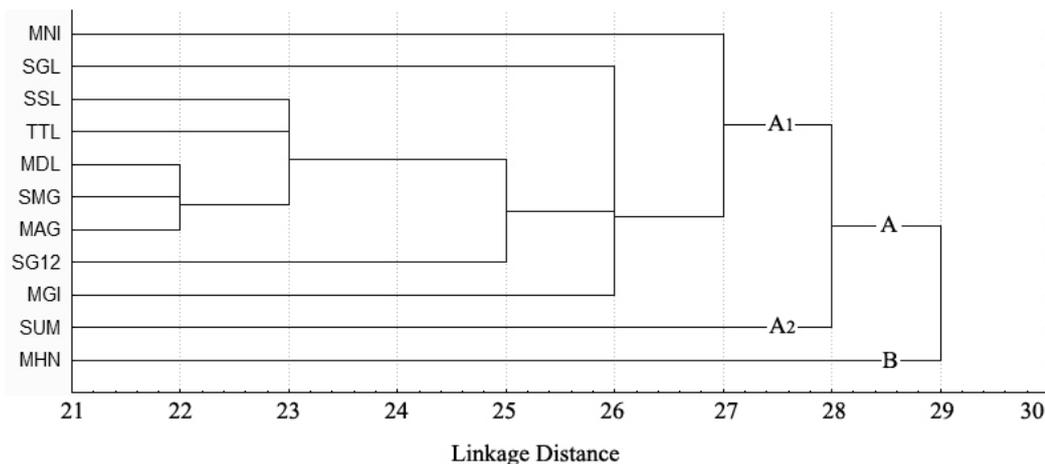


Fig. 2 Cluster analysis. Dendrogram showing RAPD-marker-based genetic relationships among 11 areca nut cultivars and grouping them into two clusters 'A' and 'B' with ten and one cultivars, respectively.

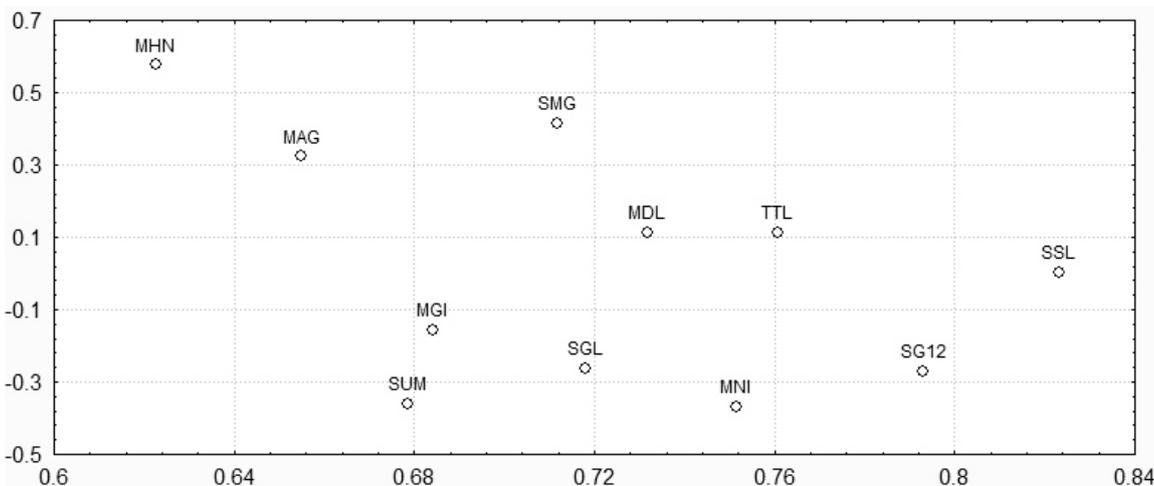


Fig. 3 PCA analysis. RAPD-marker-based genetic relationships among 11 areca nut cultivars.

The group III had only 'Mangala Intere'. The sun-cluster 'A₂' consisted of 'Sumangala' and clustered with 'A₁' at 28 linkage distances. A unique band produced by primer OPD-05 was specific to sub-cluster 'A₂'. The major cluster 'B' consisted only 'Mohit Nagar' that was linked to cluster 'A' at 29 linkage distances, and produced two polymorphic and unique bands specific to cluster 'B' according to the primers OPB-01 and OPF-08. The segregation pattern of the dendrogram was also confirmed by PCA (Fig. 3).

All the individuals in the present studies collected from the Western Ghats region of Southern India showed moderate polymorphism, despite their narrow distribution, revealing a considerable amount of phylogenetic distance among the cultivars. Since the *ex situ* collection cannot exceed a limited number of accessions, it is difficult to preserve the evolutionary potential of the species. The markers were used to determine the genetic relationship among cultivars and diversity that could have a practical application in breeding hybrids (Jain *et al.* 1999). In summary, the results from this study indicate that RAPD is a useful tool for germplasm analysis and for detection of genetic relationships within areca nut cultivars. Knowledge on genetic diversity will help in the efficient management of areca nut germplasm and hybridization programmes among breeders.

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