6 Molecular Analysis of Phylogenetic Relationships among Coconut Accessions

A. Upadhyay, J. Jose, R. Manimekalai and V.A. Parthasarathy
Central Plantation Crops Research Institute, Kerala, India

Introduction

Coconut (*Cocos nucifera* L.), a member of family *Arecaceae* is an important oil crop, grown in coastal humid tropics. It provides subsistence to millions of families in coconut growing countries. The origin of evolution of this crop is considered to be the Asia–Pacific region (Harries, 1978). Coconut accessions can be broadly classified into two types: Talls and Dwarfs. Besides other differences, the Talls are preferentially cross-pollinated whereas the Dwarfs are mainly self-pollinated. At the Central Plantation Crops Research Institute (CPCRI), India, a large collection of coconut germplasm is being maintained. India is the site for the International Coconut Gene Bank for South Asia (ICGB-SA) and extensive germplasm collecting is underway to enrich the coconut germplasm centre. Characterization and cataloguing of coconut germplasm are important for any breeding programmes to improve its productivity. At present, morphological characters are used for evaluation and classification of collected germplasm (Kumaran *et al.*, 1998). However, germplasm characterization based on morphological traits is time-consuming, expensive and sometimes unsatisfactory because of environmental effects.

Molecular markers, which detect variation at the DNA level, provide a way to characterize germplasm accurately at a faster rate. Recently, an array of molecular marker techniques has been developed. Molecular markers like restriction fragment length polymorphism (RFLP) (Lebrun *et al.*, 1998a), randomly amplified polymorphic DNA (RAPD) (Ashburner *et al.*, 1997; Rodriguez *et al.*, 1997), amplified fragment length polymorphism (AFLP) (Perera *et al.*, 1998) and microsatellites or simple sequence repeats (SSRs) (Perera *et al.*, 2000) have been successfully employed for assessing genetic diversity in coconut. Although newer techniques like AFLP and SSRs are gaining importance due to their ability to detect more polymorphism (SSRs) and high multiplex ratio (AFLP) (Powell *et al.*, 1996), RAPD markers remain popular because of their simplicity and low development cost. The RAPD technique is an efficient tool for identifying variation and estimating diversity in different biological systems (Tingey and Tufo, 1993). RAPD markers are generated by PCR amplification of random genomic segments with a single primer of arbitrary sequence (Williams *et al.*, 1990). Since no a priori knowledge of genome structure is needed, they are specially useful for analysis of less studied genomes like coconut.

In the present study, RAPD markers were used to establish the genetic similarity among some indigenous and exotic coconut accessions maintained in the coconut germplasm centre at CPCRI, Kasaragod, India and widely used in the institute's ongoing breeding programme.

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Materials and Methods

Plant material

Fourteen coconut accessions (nine Tall, four Dwarf and one intermediate type) maintained in the CPCRI germplasm collection at Kasaragod were used for the study. The details of these accessions are given in Table 6.1.

DNA extraction

DNA was extracted from newly emerged leaf using the protocol standardized earlier in our laboratory (Upadhyay *et al.*, 1999), as follows. Leaf tissue (5 g) was ground to fine powder in liquid N_2. The powdered tissue was transferred to a 50 ml polypropylene tube containing 25 ml DNA extraction buffer (100 mM Tris, 20 mM EDTA, 1% SDS, 0.2% β-mercaptoethanol, 5% PVP) and incubated at 65°C for 1h with intermittent mixing. After incubation, 15 ml phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed for 10 min by swirling. The solution was centrifuged at 20,000 g for 20 min. The supernatant was re-extracted with chloroform:isoamyl alcohol (24:1), followed by centrifugation. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 2/3 volume of isopropanol. The precipitated DNA was spooled out with a microtip, transferred to a microtube and washed twice with 76% ethanol (containing 10 mM ammonium acetate). The pellet was dried and dissolved in 1 ml TE buffer. The DNA concentration was estimated spectrophotometrically as well as by comparing the band intensity with a known quantity of DNA on ethidium bromide stained 0.8% TAE agarose gel. The average yield of high molecular weight DNA was approximately 300 $\mu g\ g^{-1}$ FW tissue.

RAPD analysis

Polymorphic primers were identified by screening 100 random decamer primers (Operon Technologies, USA) with DNA of WCT and COD. Amplification was carried out using PCR parameters as follows. The PCR reaction contained 25 ng DNA, 10 mM Tris-HCl (pH 9), 4.0 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin, 100 μM each of dNTPs, 25 pmole of primer and 1.5 U *Taq* DNA polymerase in 25 μl reaction volume. All biochemicals were procured from M/s Bangalore Genei Pvt. Ltd, Bangalore, India. DNA was amplified in DNA Engine-PTC 200 (MJ Research), programmed for denaturation at 94°C for 5 min followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. Cycling was concluded with a final extension at 72°C for a further 8 min. Amplification products were resolved by electrophoresis in a 1.2% agarose, 1X TAE gel at 60 V for 4 h. Controls lacking template DNA were included for each primer reaction mix. Amplification products were stained with ethidium bromide and visualized under UV light. Each band was considered as a RAPD marker.

Table 6.1. Details of coconut accessions.

Sl. no.	Accession	Abbreviation	Type	Place of collection
1.	West Coast Tall	WCT	Tall	Kerala, India
2.	Benaulim	BEN	Tall	Goa, India
3.	Laccadive Micro	LCM	Tall	Lakshadweep Islands, Arabian sea
4.	Laccadive Ordinary	LCO	Tall	Lakshadweep Islands, Arabian sea
5.	Kappadam	KAP	Tall	Kerala, India
6.	Andaman Ordinary	ADO	Tall	Andaman Islands
7.	Philippine Ordinary	PHO	Tall	Philippines
8.	San Ramon	SNR	Tall	Philippines
9.	Java	JVT	Tall	Indonesia
10.	Chowghat Orange Dwarf	COD	Dwarf	Kerala, India
11.	Chowghat Green Dwarf	CGD	Dwarf	Kerala, India
12.	Malayan Yellow Dwarf	MYD	Dwarf	Malaysia
13.	Malayan Orange Dwarf	MOD	Dwarf	Malaysia
14.	Gangabondam	GBD	Intermediate	Andhra Pradesh, India

Six polymorphic primers (OPA-10, OPA-11, OPB-1, OPB-5, OPC-5 and OPD-7) were selected to amplify DNA from 14 coconut accessions. The presence or absence of each PCR product was recorded.

Statistical analysis

Genetic diversity or heterogeneity was calculated according to Nei's (1975) formula. Heterogeneity was calculated for each marker and then averaged out for the total measure. Heterogeneity for Tall and Dwarf accessions was calculated by considering the markers present only in those respective groups of accessions.

The presence–absence data were entered into a binary data matrix as discrete variables (1 for the presence and 0 for the absence of a homologous band). Pairwise genetic distance was calculated based on the Nei and Li coefficient (Nei and Li, 1979) using computer package RAPDistance (Armstrong *et al.*, 1994). Genetic distance data were subjected to cluster analysis by the UPGMA method using PHYLIP software (Phylogeny Inference Package, version 3.5c, J. Felsenstein, Department of Genetics, University of Washington, Seattle).

Results and Discussion

Level of polymorphism

Of the 100 primers tested, only 54 primers amplified coconut DNA. Thirty-four primers detected at least one polymorphic band between one Tall (WCT) and one Dwarf (COD) accession. The number of polymorphic bands per primer ranged from 1 to 16. A total of 245 bands were generated by 34 polymorphic primers, of which 116 (47%) were polymorphic. The average number of polymorphic bands per primer was 2.2 (3.4 when only polymorphic primers were considered).

The number of polymorphic bands detected by each primer depends on the primer sequence, hence a variable number of polymorphic bands per primer was obtained. These results are consistent with earlier reports on RAPD analysis (Connolly *et al.*, 1994; Powell *et al.*, 1996; Ashburner *et al.*, 1997). The percentage (47%) of polymorphic bands between one Tall and one Dwarf coconut accession indicated a moderate level of polymorphism and was comparable with earlier reports on RAPD analysis in coconut (Ashburner *et al.*, 1997; Rodriguez *et al.*, 1997). The level of polymorphism in terms of the number of polymorphic bands per primer was also moderately high and found to be consistent with earlier reports on *Arabidopsis thaliana* (0.3), wheat (0.38) (Tingey and Tufo, 1993), soybean (1.56) (Powell *et al.*, 1996) and sweet potato (3.7) (Connolly *et al.*, 1994). These results indicate that RAPD markers can be a useful technique for germplasm characterization in coconut.

Polymorphism among accessions

Six primers generated 51 bands in 14 accessions, of which 35 (69%) bands were polymorphic. Among Tall accessions 50 bands were present, of which 33 (66%) were polymorphic. In contrast, Dwarf accessions had 30 bands and only 14 (47%) were polymorphic. RAPD markers were detected that were unique for LCM, LCO, BEN and WCT. The total heterogeneity among 14 accessions was 0.49 whereas that for Tall and Dwarf accessions was 0.46 and 0.40, respectively.

The pairwise genetic distance varied from 0.189 (CGD and MOD) to 0.62 (between WCT and MOD). The average genetic distance among Dwarf (0.31) was much less than that among Tall (0.45) accessions. Table 6.2 lists the genetic distance matrix for the 14 accessions.

When subjected to cluster analysis, all Dwarf accessions were grouped together (Fig. 6.1) whereas Tall accessions formed three groups. The two accessions from the Philippines, namely PHO and SNR, grouped together and along with ADO were closer to Dwarf than to Tall. KAP, a large nut sized accession from the west coastal region of India, was grouped with LCM, a small nut sized accession from the Lakshadweep Islands in the Arabian sea. Gangabondam, an intermediate type showed more genetic similarity to Dwarf accessions.

The data on genetic distance and heterogeneity indicated that more variance exists among Tall accessions than among Dwarf accessions. These results are comparable with earlier studies, which demonstrated higher variation in Tall than Dwarf (Ashburner and Rohde, 1994; Ashburner *et al.*, 1997; Perera *et al.*, 1998). This has been attributed to cross-pollination among Talls. Dwarf accessions from geographically distant regions (MOD and CGD) were genetically related (genetic distance, 0.186). Lebrun *et al.* (1998b) hypothesized that all the Dwarfs might have appeared at the same time

Table 6.2. Genetic distance matrix for 14 coconut accessions.

	WCT	PHO	BEN	ADO	LCO	JVT	KAP	SNR	LCM	COD	MOD	MYD	CGD
PHO	0.50	0.0											
BEN	0.40	0.50	0.0										
ADO	0.55	0.45	0.45	0.0									
LCO	0.49	0.53	0.43	0.44	0.0								
JVT	0.44	0.41	0.38	0.39	0.40	0.0							
KAP	0.48	0.48	0.38	0.43	0.47	0.42	0.0						
SNR	0.55	0.35	0.46	0.37	0.51	0.40	0.40	0.0					
LCM	0.45	0.55	0.39	0.53	0.54	0.50	0.32	0.51	0.0				
COD	0.60	0.45	0.48	0.30	0.47	0.38	0.49	0.40	0.55	0.0			
MOD	0.62	0.46	0.52	0.44	0.48	0.50	0.47	0.45	0.57	0.31	0.0		
MYD	0.61	0.49	0.52	0.40	0.48	0.46	0.49	0.37	0.59	0.30	0.25	0.0	
CGD	0.60	0.47	0.53	0.41	0.46	0.48	0.48	0.42	0.58	0.36	0.19	0.26	0.0
GBD	0.49	0.46	0.43	0.41	0.41	0.40	0.40	0.41	0.51	0.40	0.37	0.37	0.33

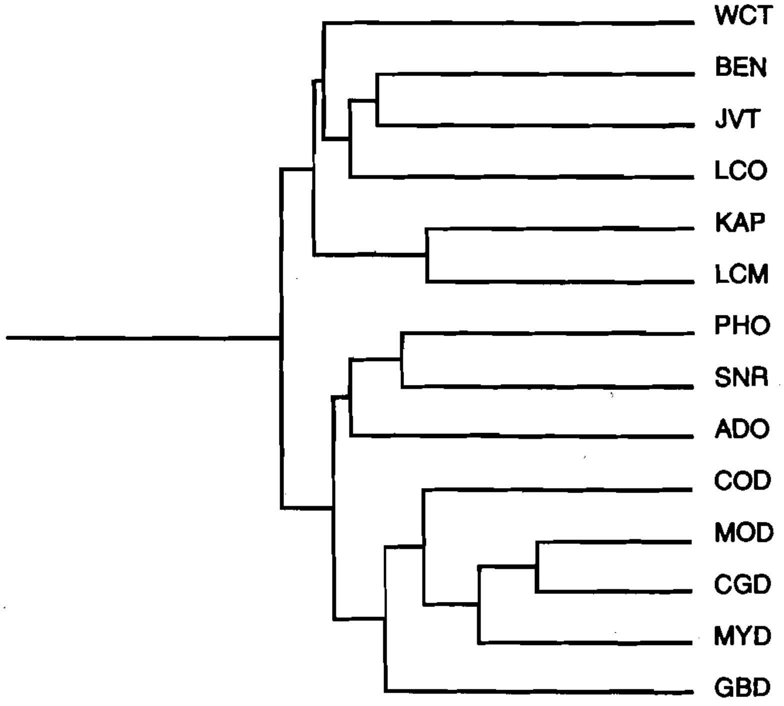

Fig. 6.1. Dendrogram showing cluster analysis of RAPD similarities among 14 coconut accessions.

and due to autogamy a major part of the genetic structure was conserved subsequently. Also Dwarf cultivars imported into new regions tend to remain genetically isolated from the local population. These reasons may explain why similar genotypes are found in distant regions.

The genetic diversity among these accessions was quite high (0.49). Ashburner *et al.* (1997) also observed high inter-population diversity among South Pacific accessions. They suggested that although the differentiation of these accessions might have arisen due to establishment of a population by a few individuals, the founder effect had not reduced diversity. The inclusion of accessions from distant regions might have resulted in relatively higher gene diversity in this study.

The cluster analysis placed two accessions with diverse fruit characters, namely KAP and LCM, together. Similar results have been reported earlier based on RFLP (Lebrun *et al.*, 1998) and phenol (Jay *et al.* 1989) analysis. The three exotic Tall collections (PHO, SNR, JVT) did not occupy a distinct position, rather, they were grouped with the indigenous accessions. Therefore, to widen genetic diversity of germplasm collections, estimation of genetic distance along with morphological data is also important.

Three Tall accessions, namely ADO, PHO and SNR, were grouped closer to Dwarf accessions. Similar results were obtained by Everard (1999) after analysing one Tall, one Dwarf and SNR. Rohde *et al.* (1995), using ISTR (Inverse Sequence Tagged Repeats) markers with 21 accessions, also found that some Tall accessions were grouped with Dwarfs. It could be assumed that grouping of the Tall accessions in this study may be due to the fact that these three accessions have some level of self-pollination due to interspadix overlapping of the mature male and female phase (Ratnambal *et al.*, 1995). The interspadix overlapping for ADO, PHO and SNR is 7.0, 4.2 and 4 days, respectively, which is comparable with intraspadix overlapping in Dwarfs. Thus the observed relationship of these accessions with Dwarfs may be due to their breeding behaviour.

In conclusion, this study has established the ability of RAPD markers to distinguish coconut accession with high efficiency. This information will form the base for analysis of intra-population variation. Extensive use of this technique and other molecular markers for characterization of coconut germplasm is envisaged. Such a study will help in planning future germplasm collecting and the selection of parents for breeding programmes.

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