Bulk Line Analysis in Coconut (*Cocos nucifera* L.) for Inferring Relationship between Talls, Dwarfs and Niu Leka Dwarf Forms

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Coconut is an important an oil crop of India. Coconut (*Cocos nucifera* L.) is botanically classified in to two major groups based on its stature as Tall palms and as Dwarf palms. The Talls can also be referred to as var. Typica (Nav) and the Dwarfs as var. Nana (Griff). Further, intermediate forms of coconut referred to as Aurantiaca are also available. The technique Bulk Line analysis is employed for the identification of tall / dwarf bulk specific RAPD marker. Thirty random primers were used to amplify the DNA bulks of talls, dwarfs and intermediate coconut types. Out of 30 primers 22 primers showed polymorphism between the DNA bulks. The primers OPM 02, OPM 06, OPC 13 produced specific markers. Out of 40 polymorphic markers five markers were common to tall bulk and dwarf bulk; on the other hand 29 markers were common between the tall bulk and Niu Leka Dwarf. To reveal the overall relationship between the bulked samples presence or absence of RAPD marker data were analysed with the NTSYS software. Jaccard's similarity coefficient was highest between talls and Niu Leka Dwarf. The resultant dendrogram showed clustering of talls and Niu Leka types together whereas, dwarf types were separated out.

Key Words: Coconut, Bulk Line Analysis, RAPD, Marker

Introduction

The coconut palm, *Cocos nucifera* Linn. is a perennial oil yielding tree crop of tropics. It belongs to the family Arecaceae under the class monocotyledons. With its tall, slender and uniformly thick stem and massive crown with large number of leaves, bearing bunches of nuts in their axils is one of the most beautiful and useful trees in the world. It perhaps yields more products of use to mankind than any other tree. Coconut has been given different names such as Kalpa Vriksha (tree of Heaven), the consols of East, Mankind's Greatest Provider in the Tropics, Tree of Life, Tree of abundance and Tree of plenty etc.

Each and every part of the coconut palm is useful to man in one way or another. The most important products are tendernut, copra, oil, oil cake, desiccated coconut and fibre. The genus *Cocos*, formerly included besides *Cocos nucifera* Linn. over thirty species mostly confined to Central and South America. It is, however, now usual to regard one of them which is cosmopolitan in the tropics as monotypic genus containing *Cocos nucifera* L. the coconut, other species having been assigned to several other new genera. Coconut has a pantropic distribution mainly in coastal regions at 20 Ú either side of the equator. Grown in 11.6 million hectares in 86 countries of tropics including India. The area and production of coconut in India (2002-03) are 1.82 M ha and 9.5 M nuts respectively (FAO STAT, 2004).

Coconuts are generally classified into tall palms and dwarf palms. Tall palms are widely planted and they can grow upto 20-30 m height. They are normally cross pollinating and therefore considered to be heterozygous. Talls constitute the polymorphic populations (Perera *et al*., 2000). They are slow to mature, flowering 6-10 years after planting and have economic life of 65-75 years. On the other hand, Dwarf palms grow to a height of 10-15 m, flowering 3-4 years after planting and they are self pollinating and considered to be homozygous and has economic life of 30-40 years. The dwarfs are presumed to have originated form talls either by mutation (Menon and Pandalai, 1958) or by inbreeding (Swaminathan and Nambiar, 1961; Lebrun *et al*., 1998). Harries (1978) recognized three basic dwarf types in coconut. They are nana, javanica and Niu Leka types. The Niu Leka dwarf coconut is characterized by its short internodes that result in a stout trunk, dense canopy and broad leaves. Harries (1978) notes that in all respects other than height, Niu Leka Dwarf resembles tall: swollen trunk base, out crossing reproductive behavior, lack of bright red and yellow fruited phenotypes and large fruit size. He suggested that Niu Leka Dwarf represented distinct domesticated types relative to other dwarf classes. Unlike the dwarf types, which are highly autogamous

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and thus homozygous, the Niu Leka Dwarf despite its name, is primarily out crossing like that Tall types (Whitehead, 1976; Harries 1978) and this is more heterozygous (Teulat *et al.*, 2000; Meerow *et al.*, 2003).

Coconut palms are identified as tall / Dwarf based on its stature. Genetically it was observed that loss of alleles and (Perera *et al.*, 2003) genetic diversity in dwarfs. But no specific markers have been identified for dwarfs / talls. Hence in the present study, the Bulk Line Analysis was employed for the identification of tall / dwarf specific markers. Earlier, Bulked Line Analysis (Tan *et al.*, 1998) was used to map the rice Rf gene. Identification of the marker for specific trait can be carried out by using various genetic stocks, such as near isogenetic lines (NILs, Muehlbauer *et al.*, 1988,), bulked segregant analysis (BSA, Michelmore *et al.*, 1991) and segregating progenies. However, all these genetic stocks are restricted to advancing generations through specific crosses. This disadvantage appears more significant for a crop having a long period for advancing a new generation like coconut. It will take 7-10 years for advancing one generation in coconut. But Bulk Line Analysis method is not based on the segregating population. The informative markers can be identified is based on the polymorphism detected by the primer between a pair of bulks having same phenotype, not between the pair of parents.

Materials and Methods

Two gram leaf samples were taken from 116 talls types, 32 dwarf types and 12 Niu Leka types. (Table 1).

DNA Extraction

DNA was extracted from sprouting leaflets (pale yellow color). Two gram of leaf material was frozen in liquid nitrogen and ground to powder in a pestle and mortar. 0.50 g of Poly Vinyl Poly Pyrrolidone (PVPP, MW 40000) was added to the ground powder. Then the DNA extraction was done using Plant DNA extraction it (Nucleon Phytopure) DNA pellet was air dried and resuspended in 100 ml TE. DNA concentration was measured in a spectrophotometer and the intactness was checked in 0.8% agarose gel. DNA was diluted to 15 ng per ml.

Bulking of DNA

Equal amounts (50 ng) of DNA samples from the 116 tall individuals were pooled to constitute the Tall Bulk. DNA samples from 32 dwarf individuals were pooled to constitute the Dwarf Bulk. DNA samples from 12 individuals of the intermediate accession (Niu Leka Dwarf) were pooled to constitute the Intermediate Bulk (Table 1). Thirty random primers were used to amplify the bulked samples

Table 1. Coconut accessions used for bulking the DNA

S.No.	Accession	Type	No. of samples
1	Kong Thienyong Tall	Tall	4
2	Straight Settlement Green Tall	Tall	4
3	Straight Settlement Apricot Tall	Tall	4
4	Philippines Kalambahim Tall	Tall	4
5	Laguna Tall	Tall	4
6	Philippines Palawan Tall	Tall	4
7	Philippines Dalig Tall	Tall	4
8	San Roman Tall	Tall	4
9	Markham Valley Tall	Tall	4
10	Nufella Tall	Tall	4
11	Nugili Tall	Tall	4
12	Nuwallis Tall	Tall	4
13	Nu Quamen Tall	Tall	4
14	Kupien Tall	Tall	4
15	Nuwehnug Tall	Tall	4
16	Lifou Tall	Tall	4
17	British Solomon Island Tall	Tall	4
18	Jamaica Tall	Tall	4
19	Saint Vincent Tall	Tall	4
20	Panama Tall	Tall	4
21	Nigerian Tall	Tall	4
22	Kaithathali Tall	Tall	4
23	Indian Spicata	Tall	4
24	Indian East Coast Tall	Tall	4
25	Verrikobbari Tall	Tall	4
26	Nadora Tall	Tall	4
27	Nicobar Tall	Tall	4
28	Hazari Tall	Tall	4
29	Navassi Tall	Tall	4
30	Niuleka Dwarf	Intermediate	12
31	Chowghat Green Dwarf	Dwarf	4
32	Laccadive Dwarf	Dwarf	4
33	Chowghat Orange Dwarf	Dwarf	8
34	Gangabondam Green Dwarf	Dwarf	8
35	Gangabondam Brown Dwarf	Dwarf	4
36	Malayan Orange Dwarf	Dwarf	4

RAPD Analysis

Amplification reactions were carried out in 10 ml volume containing 30 ng of template DNA, 200 mM of each dNTPs, 0.45 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India) and 25 pmol of random primer (Operon technologies, USA). Amplifications were performed in a Thermo Cycler (MJ Research Inc., USA) programmed for an initial denaturation at 94 °C for 2 min., 40 cycles of 1 min. denaturation at 94 °C, 1 min. annealing at 37 ºC and 2 min. extension at 72 ºC and a final extension of 7 min. at 72 ºC. The amplification products were subjected to electrophoresis through a 1.80 % agarose gel using 1X TBE buffer at 90 volts for 2 h in Bio-Rad submarine electrophoresis

unit. The ethidium bromide stained gels were documented using the Alpha Imager^{TM} 1200 – Documentation and Analysis system (Alpha Innotech Corporation, USA). Amplification reaction was done twice for reproducible bands.

Data Analysis

Only the clear, unambiguous and reproducible bands were considered for scoring. Each band was considered to be a single locus. Data were scored as "1" for the presence and "0" for the absence of a DNA band of each accession. DNA band size was estimated by comparing the DNA bands with a 1 Kb DNA ladder or lamda DNA *Eco* RI / *Hind* III double digest (MBI Fermentas, India).

The binary data matrices were entered into the NTSYS pc package (Exeter Software, USA). The data were analysed using Qualitative routine to generate Jaccard's similarity coefficient. Similarity coefficients were used to construct a dendrogram using UPGMA (Unweighted Pair Group Method with arithmetic Average) and SHAN (Sequential Hierarchical and nested clustering) routine.

Results

Bulked DNA samples of talls, dwarfs and intermediate (Niu Leka Dwarf) types were amplified with 30 RAPD primers. Out of which 22 primers produced specific markers for bulked samples (Table 2 and 3). A total of three markers were observed for tall bulk, eight markers for dwarf bulk and two markers for intermediate type. The primer OPM 02 produced one tall specific marker (OPM 02_{1425}) and one dwarf specific marker (OPM 02_{420}) and three markers common to tall bulk and intermediate bulk. The primer OPM 06 produced one dwarf specific marker (OPM 06_{1673}), two markers common to talls and dwarfs and four markers common to tall bulk and intermediate bulk. The primer OPC 13 produced Niu Leka Dwarf specific marker (OPC 13_{1320}). There were only five markers that were common to tall bulk and dwarf bulk; on the other hand 29 markers were common between the tall bulk and Niu Leka Dwarf. There were two markers OPBE 08_{1672} and OPE 02_{909} common to dwarfs and Niu Leka Dwarf. RAPD marker profiles of the bulked DNA samples with different primers are shown in Plate1.

Table 2. List of RAPD markers specific to bulked DNA samples of talls, dwarfs and intermediates

Primer	*No of specific markers (bp)*					
	Tallbulk	Dwarfbulk	Intermediate(NLAD)	Tall bulk and dwarf bulk	Tall bulk and intermediate	Dwarf bulk and intermediate
OPBE 02	0	0	0	1	3	0
OPBE 03	0	0	1	0	1	0
OPBE 06	1	0	0	0	1	0
OPBE 08	0	1	0	0	2	1
OPBE 14	0	0	0	0	2	0
OPBE 16	0	0	0	0	1	0
OPC 04	0	0	0	0	1	0
OPC 10	0	0	0	0	1	0
OPC 12	0	0	0	0	1	0
OPC 13	0	0	1	0	0	0
OPC 15	0	1	0	0	2	0
OPE 02	1	0	0	0	0	1
OPE 12	0	0	0	1	1	0
OPE 16	0	1	0	1	0	0
OPE 18	0	0	0	0	1	0
OPG 08	0	1	0	0	1	0
OPM 02	1	1	0	0	3	0
OPM 06	0	1	0	2	4	0
OPM 07	0	0	0	0	1	0
OPM 09	0	0	0	0	2	0
OPM 10	0	2	0	0	0	0
OPM 20	0	0	0	0	1	0
Total	3	8	2	5	29	2

Table 3. RAPD markers detected in the tall bulk, dwarf bulk Niu Leka Dwarf, markers common to tall and dwarf, tall and intermediate and dwarf and intermediate

Tallbulk	Dwarfbulk	Intermediate(NLAD)	Tall bulk and dwarf bulk	Tall bulk and intermediate	Dwarf bulk and intermediate
OPBE 06_{578}	OPBE 08_{920}	OPBE 03_{1320}	OPBE 02_{1233}	OPBE 02_{1451}	OPBE 08_{1672}
OPE 02_{618}	OPC_{15638}	OPC 13_{1320}	OPE 12_{854}	OPBE 02_{320}	OPE 02_{909}
OPM 02_{1425}	OPE 16_{1480}		OPE 16_{1312}	OPBE 02_{218}	
	OPG 08_{824}		OPM 06_{1121}	OPBE 03_{253}	
	OPM 02_{420}		OPM 06_{1045}	OPBE 06_{1661}	
	OPM 06_{1673}			OPBE 08_{1042}	
	OPM 10_{1758}			OPBE 08_{813}	
	OPM 10_{1440}			OPBE 14_{1202}	
				OPBE 14_{987}	
				OPBE 16_{1412}	
				OPC 04_{440}	
				OPC 10_{472}	
				OPC 12_{558}	
				OPC 15_{617} OPC 15_{602}	
				OPE 12_{442}	
				OPE 18_{583}	
				OPG 08_{478}	
				OPM 02_{1623}	
				OPM 02_{672}	
				OPM 02_{646}	
				OPM 06_{1741}	
				OPM 06_{1454}	
				OPM 06_{908}	
				OPM 06_{602}	
				OPM 07_{592}	
				OPM 09_{1077}	
				OPM 09_{889}	
				OPM 20_{493}	

To reveal the overall relationship between the bulked samples, the presence / absence of RAPD marker data were analysed using the software NTSYS pc. 30 primers produced a total of 291 markers. Out of which, 40 markers were polymorphic. Jaccard's similarity coefficient between the tall bulk and the dwarf bulk was 0.819, between the tall bulk and Niu Leka Dwarf was 0.935, between the dwarfs and Niu Leka Dwarf was 0.810. The dendrogram grouped the tall bulk and Niu Leka Dwarf together and the dwarf bulk was separated out (Fig. 1).

Discussion

The utility of bulk line analysis (Tan *et al.*, 1998) was tested in coconut for the identification of dwarfs / talls palms. BLA method is not based on the segregating population and the informative markers identified is based on the polymorphism detected by the primer between a pair of bulks, not between the pair of parents.

Based on the method of BLA, RAPD markers were identified that were specific to talls and dwarfs. Eight RAPD markers were identified for dwarfs. The interesting observations was, most of tall bulk markers were shared by the intermediate type, Niu Leka Dwarf. This again confirms the Niu Leka Dwarf is not a true dwarf type and it is intermediate between talls and dwarfs and more close to talls. Highest similarity found between talls and Niu Leka Dwarf reveals the closeness between them. Hence, our data suggest that, Niu Leka Dwarf can be placed towards the talls. Dendrogram constructed revealed the overall relationship among talls, dwarfs and Niu Leka Dwarf. Meerow *et al.* (2003) reported Niu Leka Dwarf had the closest genetic identity with the Panama Tall and Spicata Tall and had largest number of unique SSR alleles. Our data revealed that the Niu Leka Dwarf shared most of the markers to the talls and clustered with the talls.

The DNA markers identified based on the bulked samples has immense tool for juvenile selection of tall / dwarf at early stages among hybrids of tall x dwarf or dwarf x tall. There was no such standard molecular

level guidelines available so far for the identification of seedlings of talls and dwarfs at nursery. But, some available discriminators *viz*., time taken for sprouting, rate of leaf production, length and width of leaves and time to first leaf splitting, needs six months to one year to observe these characters. Moreover, all traits are influenced by environment and require proper field experiments. Fernando *et al*. (1993) used seedling characteristics to identify the hybrids and they reported 20 per cent chance of misclassification. But, when DNA markers are available seedling can be identified unambiguously. Microsatellite markers would be better a tool for bulk line analysis also. The effectiveness of these markers needs to be tested in the field conditions.

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