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Genetic diversity of coconut (*Cocos nucifera* L.) in Sri Lanka revealed by randomly amplified polymorphic DNA (RAPD) markers

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Abstract

Genetic diversity of seventeen germplasm accessions and three improved cultivars was determined by RAPD analysis. Twenty Operon primers selected from 100 primers were used and these primers amplified coconut DNA generating 129 amplification products averaging 6.5 bands per primer. Among them 84 (65.12%) exhibited polymorphoisms averaging 4.2 polymorphic bands per primer. The Nei and Li pair-wise genetic distance matrix revealed genetic base in the coconut in Sri Lanka with distances ranging from 0.036 – 0.252 with an average distance of 0.145. The 20 coconut types assayed clustered into two groups. The first included all dwarf forms, aurantiaca form King coconut, and San Ramon. The other comprised typica forms and the three improved cultivars .San Ramon grouped with dwarf types exhibiting its evolutionary link with dwarfs as Philippine coconuts. King coconut too indicated a South East Asian Origin similar to dwarfs. As expected improved cultivars grouped with tall types as all these cultivars have a tall as one parent.

Key words: coconut, RAPD, genetic diversity

1. Introduction

Coconut is one of the most important plantation crop in the wet tropics. Its food and industry based products play a very important role in the economics of many developing countries. In Sri Lanka 21% of the total land under agriculture is cultivated with coconut. More importantly, coconut is an important constituent of the daily diet of the average Sri Lankan providing approximately 25% of the calorific requirement (David, 1984).

The coconut in Sri Lanka was categorized into three distinct varieties, tall (*typica*), dwarf (*nana*) and thembili or king coconut (*aurantiaca*). Tall coconuts are the most

commonly grown and commercially exploited. They are predominantly cross-pollinating, late bearing and producing nuts that are from medium to large in size. Dwarf coconuts, mostly grown for ornamental and breeding purposes are predominantly self-pollinating. Dwarf coconuts produce small nuts in large quantities with distinct colour forms. The intermediate, thembili or king coconut is also self-pollinating and the sweet nut water characterizes this variety (Liyanage 1958). Fifteen forms have been identified within these three varieties.

Coconut Research Institute (CRI) of Sri Lanka is the main supplier of seed coconuts and has been able to produce three varieties with over 75 years of research. CRI field gene banks have more than 100 accessions of germplasm collected from many parts of the country as well as by other means such as phenotypically distinctive form, exotic forms, drought tolerant etc. Coconut breeding is difficult, costly and time consuming due to its long juvenile phase and large size of the crop. It is impossible to evaluate crosses of all combinations of germplasm available in gene banks. Therefore it is important to identify the genetical structure of these coconut germplasm accessions for the prioritization of crosses.

Morphological descriptors use for characterizing germplasm are inaccurate and could give falsified information due to large environmental effects on their manifestation. Characterizing these materials at the DNA level is extremely convenient and likely to give a more accurate description of genetic relationships. This paper reports assessment of the three improved cultivars and 17 accessions of coconut germplasm conserved *ex-situ* at the CRI of Sri Lanka using a DNA based technique RAPD.

2. Materials and methods

Plant material

Plant materials were obtained from the *ex-situ* conserved coconut germplasm in field gene banks of coconut Research Institute (CRI) of Sri Lanka. Three improved cultivars (Tall x San Ramon, Dwarf Green x Tall and Dwarf Yellow x Tall) and 17 accessions were selected for the assessment. These seventeen accessions comprised 11 tall or typica forms (Tall Ambakelle, Gon Thembili, St Ann's, Moorock, Palugaswewa, Goyambokka, Ambakelle special, Kasagala, Debarayaya, Margaret and San Ramon); 5 dwarf or nana forms (Dwarf Yellow, Dwarf Green, Dwarf Brown, Cameroon Red Dwarf and Brazilian Green Dwarf) and 1 thembili or aurantiaca form (King coconut).

DNA extraction

For each accession, 20 individuals were pooled for extraction of DNA. DNA was extracted using a CTAB based protocol developed from Doyle and Doyle (1990) by

the authors. The immature leaf material (3 g) was ground in liquid nitrogen and incubated with 15 ml of preheated (60°C) CTAB buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0) 10mM Tris-HCl (pH 8.0) and 0.2% β mercaptoethanol added just prior to dispensing the sample] at 60°C for 1 h. After two chloroform extractions the aqueous phase was separated and treated with RNAse A (50 mg/ml) at 37°C for one hour. The DNA was precipitated by adding 0.6 volumes of cold isopropanol to the sample and collected by hooking with a bent glass rod. The fibrous DNA was washed by gently agitating in 20 ml of washing solution (76% ethanol and 10 mM ammonium acetate) and pelleted by centrifugation (5000g, 10 minutes at 4°C). The pellet after suspending in 2 ml of TE buffer was treated with 0.5 volumes of 7.5 M ammonium acetate in an ice slurry for 15 minutes. The supernatant after centrifugation (10000 g, 30 minutes at 4°C) was separated and DNA was precipitated by adding 2 volumes of ethanol. The pellet was washed in 70% ethanol and collected by centrifugation (13000 rpm, 10 minutes at 4°C). After leaving in the air for drying for about an hour the pellet was suspended in 500 µl of TE.

Detection of RAPD profile

Extracted DNA was diluted to a final concentration of 15 ng/µl in ultra pure water (Sigma) and used in single primed polymerase chain reaction (RAPD-PCR) to generate random amplified polymorphic DNA (RAPD) profile.

For RAPD-PCR studies one hundred randomly generated 10-mer primers were used in single primer PCR. The primers were purchased as kits OPA (OPA01-20), OPB (OPB01-20), OPC (OPC01-20), OPD (OPD01-20) and OPE (OPE01-20) from the Operon Technologies Limited, USA.

The reaction condition used for assaying RAPDs with coconut DNA were very much similar to the original protocol described by Williams *et al* (1990). For PCR approximately 75 ng of genomic DNA was amplified in 25 µl volumes containing 1xPCR buffer (50 mM potassium chloride, 10 mM Tris-HCl pH 9.0 (at 25°C), 0.1% Triton x-100) supplied with enzyme (Promega), 1.8 mM $MgCl_2$ supplied with enzyme (Promega), 0.16 mM dNTP (Pharmacia), 10 pmol random primer (Operon Tech. USA) and 1 unit of *Taq* polymerace (Promega). Each reaction mixture was overlaid with a drop of mineral oil. Amplification was achieved in PTC100-thermocycler (MJ Research, Inc) for 45 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C.

RAPD-PCR was conducted with all the 100 primers using DNA from Tall, Dwarf Green and San Ramon, the three parent types currently used in production of coconut hybrids. Among them 20 primers were selected on the basis of their ability to produce consistent amplifications with coconut DNA. The RAPD assay was carried out for the 20 germplasm accessions of coconut with these 20 primers.

Detection of polymorphisms

Once the thermal cycling had finished, 3 µl of loading buffer were added to the 25 µl of reaction mix and mixed well by giving a small pulse. Aliquots of 18 µl were then loaded on to 1.5% agarose gel and run in 1xTAE buffer or TEB buffer for approximately 3.5 hours at 5 V/cm (at constant voltage of 40 V) and detected by ethidium bromide (0.5 µg/ml) staining.

The stained gels were scanned under UV by Photo-Print Gel Documentation system (Vilber Lourmat) and the images were captured and scored for presence (1) or absence (0) of bands in individuals and the accurate size of each fragment was estimated by comparing with the DNA standard, 1-kb ladder (Sigma) aided by Photo-Capt software supplied with the gel documentation system.

Data analysis

The genetic relatedness of the DNA samples was calculated by pair-wise comparison that based on band sharing. Initially a genetic distance matrix was constructed using the software RAPDistance developed by J.Armstrong of Australia National University, Canbera. The distances were computed using the Nei and Li's (1979) formula. The distance matrix was subsequently used in the same software and constructed the dendrogram to depict the genetic relatedness of the DNA samples.

3. Results and discussion

Twenty Operon primers were tested on 20 coconut types of Sri Lanka. Figure 1 shows RAPD profile obtained by primer OPB07. These 20 primers amplified a total of 129 fragments averaging 6 – 7 per primer. Out of 129, 84 showed polymorphisms. The size of amplified fragments ranged between 200 bp to 2.45 kbp. All primers detected polyhmorphisms averaging 65%, ranging from 40% (with OPOD3 and OPD19) to 100% (with OPA12 and OPE12) per primer (Table 1).

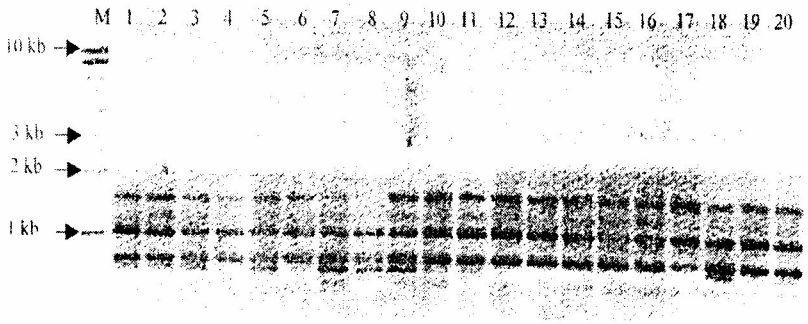

Figure 1: Random amplified polymorphic DNA profile of 20 coconut germplasm accessions conserved *ex-situ* in Sri Lanka generated by primr OPB07. Lane M is 1 kb ladder maker. Lanes 1-20 are TT (Tall Ambakelle), AS (Ambakelle special), KS (Kasagala), SR (San Ramon), MG (Magaret), MK (Moorock), DEB (Debarayaya), STA (St. Ann's), GB (Goyambokka), PG (Palugaswewa), GT (Gon thebili), KC (King coconut), DY (Dwarf Yellow), DG (Dwarf Green), DB (Dwarf Brown), CRD (Cameroon Red Dwarf), BGD (Brazilian Green Dwarf), TSR (Tall x San Ramon), DYT (Dwarf Yellow x Tall), DGT (Dwarf Green x Tall).

Genetic distances among the accessions ranged from 0.036 – 0.252 (Table 2). Lowest distance was found between Dwarf Brown and Dwarf Green; Cameroon Red Dwarf and Dwarf Brown. Maximum distance (0.252) was observed between Brazilian Green Dwarf and Kasagala. Brazilian Green Dwarf was the most deviant of all types averaging distances 0.23 and 0.13 with tall and dwarf. Overall average distance was 0.145, indicating little genetic diversity existing coconut in Sri Lanka. Average genetic distance between tall and dwarf accessions was 0.2. Genetic distance between San Ramon and Tall, and San Ramon and Dwarf was 0.189 and 0.125 respectively which indicate that San Ramon is more close to Dwarf than the Sri Lanka Tall (Table 3).

The dendrogram based on genetic distances (Figure 2) depicted the genetic relatedness of the accessions by clustering into two major groups. The first comprised all dwarf forms, aurantiaca form King coconut, and San Ramon. According to the earlier classification King coconut (aurantiaca) form is an intermediate form between tall and dwarf forms. But here King coconut has grouped with dwarf forms. Even most of the morphological (slender trunk) and reproductive characters (self-pollinating and profuse bearing capacity) of King coconut also place it more towards a dwarf than a typical Sri Lanka tall. Similar observations have also been reported by Perera *et al* (1998 and 2000) through variations detected by amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs).

Striking difference in the whole cluster is grouping of San Ramon with dwarfs. Although San Ramon is a typica (tall) form in morphological features it has a different origin as it is introduced from Philippine. The Sri Lanka talls resemble African tall coconuts.

Table 1. Details of the 10-mer oligonucleotides

Primer	Sequence (5'-3')	No of Amplification Products	No of Polymorphic Products	Range of band size (kb)	% Polymorphi -sms
OPA03	AGTCAGCCAC	03	02	0.85-2.0	66.6
OPA09	GGGTAACGCC	08	06	0.20-1.1	75.0
OPA10	GTGATCGCAG	03	02	0.40-0.8	66.6
OPA12	TCGGCGATAG	06	06	0.60-1.2	100.0
OPA16	AGCCAGCGAA	07	05	0.50-2.4	71.4
OPA20	GTTGCGATCC	03	02	0.65-1.5	66.6
OPB04	GGACTGGAGT	06	04	0.25-1.8	66.6
OPB05	TGCGCCCTTC	07	04	0.60-1.7	57.1
OPB07	GGTGACGCAG	06	03	0.50-1.5	50.0
OPB10	CTGCTGGGAC	07	03	0.70-2.3	42.8
OPC07	GTCCCGACGA	10	08	0.60-2.0	80.0
OPC08	TGGACCGGTG	07	04	0.50-1.9	57.1
OPC10	TGTCTGGGTG	08	06	0.60-1.7	75.0
OPC13	AAGCCTCGTC	08	04	0.60-2.1	50.0
OPC17	TTCCCCCCAG	05	03	0.50-1.7	60.0
OPD03	GTCGCCGTCA	05	02	0.70-1.3	40.0
OPD12	CACCGTATCC	07	04	0.70-1.9	51.1
OPD19	CTGGGGACTT	05	02	0.50-1.3	40.0
OPE02	GGTGCGGGAA	09	05	0.10-2.2	55.5
OPE12	TTATCGCCCC	09	09	0.30-1.5	100.0
		129	84		

	TT	AS	KS	SR	MG	MK	DEB	STA	GB	PG	GT	KC	DY	DG	DB	CRD	BGD	TSR	DYT	DGT
TT (Tall Ambakelle)	0.0																			
AS (Ambakelle special)	0.042	0.0																		
KS (Kasagala)	0.105	0.082	0.0																	
SR (San Ramon)	0.180	0.196	0.196	0.0																
MG (Magaret)	0.105	0.103	0.154	0.137	0.0															
MK (Moorock)	0.075	0.084	0.126	0.210	0.105	0.0														
DEB (Debarayaya)	0.057	0.087	0.117	0.180	0.128	0.130	0.0													
STA (St. Ann's)	0.074	0.083	0.125	0.168	0.104	0.127	0.077	0.0												
GB (Goyambokka)	0.059	0.079	0.132	0.206	0.111	0.081	0.105	0.090	0.0											
PG (Palugaswewa)	0.071	0.069	0.133	0.218	0.112	0.071	0.117	0.113	0.076	0.0										
GT (Gon Thembili)	0.073	0.092	0.113	0.196	0.134	0.105	0.097	0.083	0.111	0.080	0.0									
KC (King coconut)	0.181	0.177	0.167	0.120	0.167	0.191	0.181	0.179	0.197	0.200	0.177	0.0								
DY (Dwarf Yellow)	0.202	0.208	0.218	0.108	0.166	0.202	0.212	0.210	0.219	0.210	0.218	0.056	0.0							
DG (Dwarf Green)	0.202	0.208	0.208	0.218	0.177	0.223	0.202	0.189	0.208	0.221	0.197	0.056	0.063	0.0						
DB (Dwarf Brown)	0.206	0.202	0.191	0.113	0.181	0.227	0.206	0.193	0.223	0.225	0.202	0.051	0.068	0.036	0.0					
CRD (Cameroon Red Dwarf)	0.191	0.197	0.177	0.118	0.166	0.212	0.191	0.178	0.208	0.200	0.187	0.046	0.084	0.052	0.036	0.0				
BGD (Brazilian Green Dwarf)	0.224	0.241	0.252	0.166	0.197	0.247	0.213	0.222	0.231	0.245	0.241	0.135	0.133	0.133	0.116	0.122	0.0			
TSR (Tall x San Ramon)	0.150	0.147	0.157	0.149	0.106	0.181	0.121	0.128	0.156	0.178	0.147	0.130	0.158	0.128	0.132	0.138	0.145	0.0		
DYT (Dwarf Yellow x Tall)	0.117	0.104	0.135	0.188	0.114	0.148	0.119	0.105	0.133	0.135	0.125	0.117	0.168	0.136	0.141	0.136	0.188	0.056	0.0	
DGT (Dwarf Green x Tall)	0.145	0.153	0.163	0.155	0.112	0.187	0.137	0.144	0.162	0.185	0.153	0.115	0.144	0.113	0.117	0.123	0.152	0.045	0.061	0.0

Table 2. The Nei and Li pair-wise genetic distance matrix based on randomly amplified polymorphic DNA (RAPD) band sharing of 20 coconut types conserved *ex-situ* in Sri Lanka.

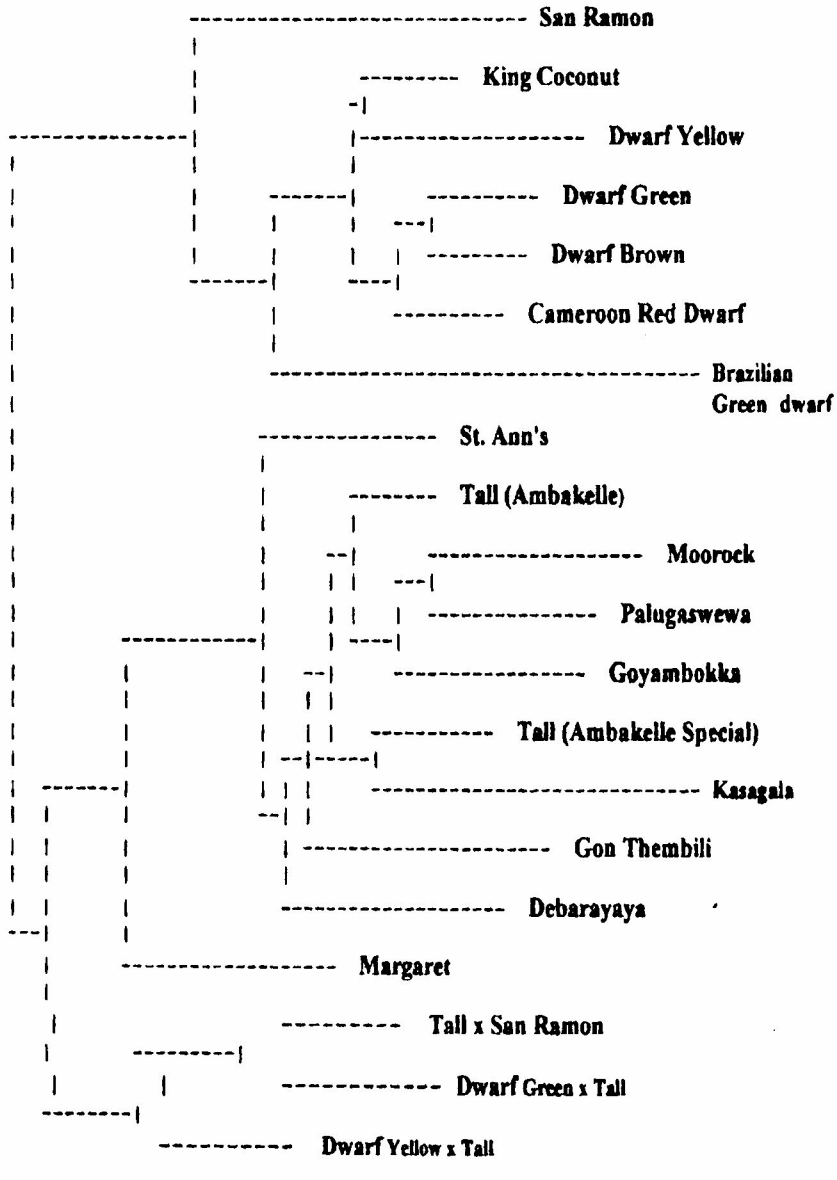

Figure 2. Dendrogram showing genetic relatedness of 17 coconut accessions and three improved cultivars of coconut in Sri Lanka

Table 3. Genetic distances between the varieties.

	Genetic distances
Overall	0.145
Within tall	0.114
Within dwarf	0.11
Between tall and dwarf	0.2
Between San Ramon and tall	0.189
Between San Ramon and dwarf	0.125
Between Brazilian Green Dwarf and tall	0.23
Between Brazilian Green Dwarf and dwarf	0.13

Therefore this unusual clustering of San Ramon clearly indicate the common genome it has with dwarf which is more characteristic of South East Asian coconuts.

The second group consisted all tall types and hybrids. Further sub clustering of this group separated hybrids. The close clustering of all Sri Lanka tall accessions indicates that they are closely related and domesticated from a common introduction. This introduction is thought to be from India or Africa as they bear similar features such as moderate trunk , height and elongated average size nuts. The genetic relatedness of Sri Lanka tall, Indian tall and African tall was confirmed by previous studies of Perera *et al* (2000) with SSRs, Teulat *et al*., (2000) by SSRs and AFLPs and Lebrun *et al*., (1998) by RFLP (Restriction Fragment Length Polymorphisms) markers. The accessions in the second sub cluster were hybrids (Tall x San Ramon, Dwarf Green x Tall and Dwarf Yellow x Tall). The assembling of hybrids with tall types is expected as all the three cultivars have tall as one parent.

Accession Margaret has separated from other tall types. Perera *et al* have made similar observations in 2001 with SSR assessment. It has been reported that original populations of Margaret is exotic and is also morphologically different from other tall types. It resembles the variety San Ramon in their nut shape and trunk characteristic.

There are three verities of coconut in Sri Lanka based on their morphological differences. But only two groups could be identified based on genomic differences.

Dwarf forms, King coconut and San Ramon falls into first group and this group resembles coconuts of pacific region. They have large round shaped nuts. Ecotypes and forms of Sri Lanka tall fall into second group and it resembles coconuts of the Indo-Atlantic region. They have medium sized elongated nuts with much slender trunks.

The genetic relationships determined by RAPDs are generally consistent with previous studies (Everard 1996, Perera *et al* 2001). With exceptions of San Ramon, Cameroon Red Dwarf, Brazilian Green Dwarf and Margaret all other genotypes studied are indigenous to Sri Lanka. But these introduced varieties did not occupy more genetically distant positions in the dendrogram. It highlights need of characterization of germplasm at the DNA level than using morphological markers, which are mostly affected by environmental factors.

In summary the genetic relatedness of the coconut in Sri Lanka was accurately explained by the RAPD assay. The coconut germplasm conserved *ex-situ* in the Coconut Research Institute has a narrow genetic base indicating fewer chances for substantial genetic improvement. The two accessions somewhat distant being exotic types, Brazilian Green Dwarf and San Ramon suggest the possibility of introducing exotic coconuts for gene pool enrichment.

4. Acknowledgement:

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