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Characterization of Coconut Germplasm by Microsatellite Markers

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ABSTRACT: *Seventeen coconut specific microsatellite or simple sequence repeat (SSR) primers were used for characterizing the coconut germplasm in Sri Lanka. A sample of thirty accessions from a pool of approximately 100 ex situ conserved coconut accessions was screened and the primers detected 75 alleles ranging 2 to 8 alleles/locus. The genetic distances among the accessions ranged between 0.13 and 1.0. The high values of genetic distances clearly reflect the ability of SSR to identify polymorphisms in coconut DNA. The dendrogram constructed from the genetic distances separated the 30 accessions into two major groups. The first comprising all dwarf, semi tall and semi dwarf and king coconut phenotypes, Bodiri and Philippine coconut ecotypes. The second group with Sri Lanka Tall (SLT) coconut ecotypes and four others slightly differs from morphological features to SLT. This is a clear demonstration of separating coconut accessions based on genetical relationship attributed to geographical distribution and ancestry. The members of the first sub group shared the South East Asian or Pacific genome while the accessions in the second group shared the Indo Atlantic or African tall genome. Further, sub clustering of the groups discriminated more heterozygous phenotypes within the groups such as those other than pure dwarf type coconut accessions from the rest in the group one and those other than pure Sri Lanka tall type coconut accessions and the rest in group two. The results demonstrated the ability of coconut specific SSR primers to characterize the coconut germplasm. These results are also helpful in efficient selection of parents for coconut breeding programmes and identification of duplicates and core populations of the gene pool for effective conservation and management of germplasm.*

INTRODUCTION

Coconut (*Cocos nucifera* L.) plays a very important role in the economics of many developing countries, including Sri Lanka, where 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). Coconut has a prominent place among many perennial crops in the world because of its multitude of uses. International Plant Genetic Resources Institute has created a separate international body, Coconut Genetic Resources Network (COGENT) to ensure conservation, characterization and utilization of coconut biodiversity throughout the world.

The genetic diversity of coconut in Sri Lanka is little known except for few

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phenotypic variants. The coconut in Sri Lanka was categorized into three distinct varieties, tall (*typica*), dwarf (*nana*) and *Thembili* or king coconut (*aurantiaca*) (Liyanage, 1958). 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 (Liyanage, 1958). In general, tall coconuts are hardy and can thrive in a range of environmental conditions. Dwarf coconuts, mostly grown for ornamental and breeding purposes are predominantly self-pollinating and less adapted for harsh environments. Dwarf coconuts produce small nuts in large quantities with distinct colour forms. The intermediate, *Thembili* or king coconut is also autogamous and the sweet nut water characterizes this variety (Liyanage 1958).

Many intra-varietal and inter-varietal coconut hybrids have been produced in coconut growing countries. The coconut hybridization programmes are aimed at searching heterosis for traits such as early flowering and bearing, more nuts and copra and adaptability to a range of agro-climatic conditions. To develop new cultivars; breeders require a wide collection of germplasm constituting genetically diverse accessions to breed and test for heterosis in such traits. *Ex situ* field gene banks of the Coconut Research Institute (CRI) of Sri Lanka hold a germplasm repository of about 100 accessions collected from various locations island wide. These collections mostly constitute tall coconuts, which are phenotypically same. In addition, these gene banks have various dwarf collections and a few phenotypically distinct coconuts collected from home gardens but of unknown origin.

The progress of coconut breeding by conventional methods alone was constrained by long juvenile phase, time and cost limitations, lack of vegetative propagation methods, small number of seed produced per year and the inherent heterozygosity. More accurate characterization of coconut germplasm using molecular markers appears as a better alternative for selecting diverse coconut accessions for prospective heterosis breeding exercises.

Molecular markers have already been used in characterizing coconut germplasm in Sri Lanka and in other countries (Everard *et al.*, 2000, Perera *et al.*, 2000, Ashburner *et al.*, 1997). More recently COGENT has tested the potential of coconut specific microsatellite primers for characterization of coconut germplasm. Microsatellites or simple sequence repeats polymorphisms ($SSRP_s$) are now the choice of many for assessing germplasm because of the SSR's high abundance, high rate of polymorphisms, co-dominant inheritance and suitability for automation. The objective of this study was to characterize a representative sample of coconut germplasm conserved *ex situ* at the CRI, using SSR polymorphism.

MATERIALS AND METHODS

Plant material

Plant materials were obtained from the *ex situ* conserved coconut germplasm in field gene banks of CRI of Sri Lanka. Among approximately 100 *ex situ* conserved germplasm, 30 accessions were selected for the assessment. These 30 accessions comprised 17 distinct phenotypes [6 tall (*typica*), 8 dwarf (*nana*) and 3 *Thembili* (*aurantiaca*) forms], 6 San Ramon tall like ecotypes and 7 Sri Lanka Tall ecotypes.

DNA extraction

For each accession, 20 individuals were pooled for extraction of DNA. DNA was extracted by using a CTAB based protocol developed from Doyle and Doyle (1990) by Dasanayake *et al.*, 1999. The immature (yellow colour) leaf material (3 g) was ground in liquid nitrogen and incubated with 15 ml of preheated (60^{0}C) CTAB buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0) 10 mM Tris-HCl (pH 8.0) and 0.2% β mercaptoethanol added just prior to dispensing the sample] at 60^{0}C for 1 h. After two chloroform extractions the aqueous phase was separated and treated with RNAse A (50 mg ml^{-1}) at 37^{0}C for 1 h. 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 (5000 g, 10 minutes at 4^{0}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^{0}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^{0}C). After leaving in the air for drying for about an hour the pellet was suspended in 500 µl of TE.

Detection of microsatellite polymorphisms

Seventeen microsatellite primers, pre-selected from a pool of 35 primers [developed by Perera *et al.* (1999) and Rivera *et al.* (1999)] were used for detecting simple sequence repeat polymorphisms (SSRPs) in the coconut samples. PCRs were performed in 10 µl volumes with 1 µM forward primer, 1 µM reverse primer, 1 unit of *Taq* DNA polymerase (Promega), 0.2 mM each of dNTP (Pharmacia), 1 x PCR buffer [50 mM potassium chloride, 10 mM Tris-HCl pH 9.0 (at 25^{0}C), 0.1% Triton x-100)] supplied with the enzyme (Promega), 1.5 mM $MgCl_2$ supplied with the enzyme (Promega) and 30 ng of template DNA in a PTC-100 Thermocycler (MJ Research, Inc) with 30 thermal cycles, 1 minute denaturation at 94^{0}C, 1 minute annealing at 51^{0}C-58^{0}C (depending on primer) and 1 minute primer extension at 72^{0}C. The first cycle was preceded by a 3 minutes denaturation at 95^{0}C, and the last cycle followed by a 2 minutes extension at 72^{0}C. Reaction products were electrophoresed on 6% denaturing polyacrylamide gels and visualized by staining with silver nitrate.

Data analysis

The fragments amplified by microsatellite primers were scored as present (1) and absent (0). The genetic relatedness of the DNA samples was obtained by calculating Nei and Li (1979) pair-wise genetic distances and by construction of a dendrogram using the software "RAPDistance" developed by J. Armstrong of Australia National University, Canberra, Australia.

RESULTS AND DISCUSSION

The 17 SSR primers identified 75 alleles with an average of 4.4 alleles per locus ranging from 2 (CAC56) to 8 (CAC50). All the primers detected polymorphism in the 30 genotypes assessed (Table 1). Perera *et al.* (2000) has used eight pairs of SSR primers to analyze the genetic diversity in 130 individuals of coconut representing 94 different coconut ecotypes throughout the world. In this study a total of 51 alleles were detected at an average of 6.4 alleles per locus ranging from 3 to 9 alleles per locus.

Teulat *et al.* (2000) has assessed genetic diversity in 31 individuals from 14 coconut populations across the entire geographic range with 37 SSR primers. They have detected 339 alleles at an average of 9.4 alleles per locus ranging from 2 to 16 alleles per locus. This higher number of alleles per locus in those two latter cases was obviously due screening of coconut ecotypes throughout the world.

Table 1. SSR repeat type, allele size and number of alleles per locus for the SSRs coconut accessions.

Locus	Type of repeat	Size of the product (bp)	No. of alleles
CAC06	$(AG)_{14}(CA)_9$	158	5
CAC08	$(AG)_{10}(CA)_9$	190	5
CAC23	$(CA)_8$	192	3
CAC38	$(CA)_{13}(CT)_{17}$	155	5
CAC50	$(TA)_9(CA)_{21}$	153	8
CAC56	$(CA)_{14}$	154	2
CAC65	$(CA)_{15}$	151	3
CAC68	$(CA)_{13}$	142	5
CAC77	$(CA)_{15}(CT)_{11}$	131	4
CNZ04	$(CT)_{29}TT(CA)_{10}$	162	5
CNZ06	$(CT)_{15}$	85	3
CNZ10	$(CT)_{18}(GT)_{17}$	148	5
CNZ12	$(CT)_{15}$	214	3
CNZ29	$(GT)_{22}(GA)_2CA(GA)_{11}$	135	4
CNZ43	$(GA)_{21}$	197	7
CNZ44	$(GA)_{15}$	165	4
CNZ46	$(CT)_{24}$	116	4
Average	-	-	4.4

Genetic distances among the accessions ranged from 0.13 to 1.0 with an average of 0.54 (Table 2). Lowest distance was found between Dwarf green and Mirishena dwarf; Cameroon red dwarf and Mirishena dwarf and Cameroon red dwarf and dwarf green. This indicates the narrow genetic base in the dwarf coconuts than the other coconuts. Maximum distance (1.0) was observed between 16 pairs. Among them 15 were between tall and dwarf accessions and one was between Clovis (Philippine coconut) and Maliboda (tall accession). This high distance was observed due to sharing of completely different alleles between two accessions within a pair. Perera *et al.* (2000) also found certain microsatellite alleles in dwarf coconut completely absent in Sri Lanka tall accessions. This clearly indicates that dwarf coconuts in Sri Lanka represent a separate introduction very much unrelated to Sri Lanka tall. The high values of genetic distances obtained with simple sequence repeat polymorphisms clearly indicated the highly polymorphic nature of SSR loci.

The dendrogram based on genetic distances (Figure 1) depicted the genetic relatedness of the accessions by clustering into two major groups. The first comprised all dwarf forms, semi tall and semi dwarf (Mirishena), king coconut forms, Bodiri and the two Philippine coconuts: Clovis and Nipuni. Further sub clustering of this group separated more heterozygous types such as semi tall, Clovis, Nipuni and Bodiri. These results confirm a common putative origin of dwarf coconut and the Philippine tall coconuts; Clovis and Nipuni. The latter is a collection obtained from a single palm in

Table 2: The Nei and Li pair-wise genetic distance matrix based on microsatellite band sharing ratio of 30 coconut germplasm accessions conserved *ex-situ* in Sri Lanka

	MST	DIC	DAD	NIP	IND	SR-R	AKU	KC	NWT	MAL	LAN	BGD	BLA	RT	BOD	DB	KAM	GT	PP	NAW	DR	DY	MID	RT	DG	KAS	DEB	CLO	CRD	DAM
Mirishena ST (MST)	0.00																													
Dickwella (DIC)	0.65	0.00																												
Dadalla (DAD)	0.86	0.67	0.00																											
Nipuni (NIP)	0.46	0.57	0.60	0.00																										
Indian (IND)	0.56	0.46	0.46	0.50	0.00																									
San ramon RT (SR-RT)	0.63	0.46	0.51	0.63	0.18	0.00																								
Akuressa (AKU)	0.65	0.68	0.50	0.62	0.44	0.62	0.00																							
King coconut (KC)	0.46	0.77	0.79	0.49	0.69	0.74	0.71	0.00																						
Nawasi thembili (NWT)	0.64	0.84	0.71	0.47	0.67	0.70	0.67	0.35	0.00																					
Mafiboda (MAL)	0.90	0.70	0.65	0.79	0.64	0.72	0.28	0.85	0.74	0.00																				
Lanlib (LAN)	0.76	0.64	0.53	0.70	0.43	0.57	0.29	0.89	0.68	0.37	0.00																			
Brazilian GD (BGD)	0.41	0.66	0.74	0.53	0.69	0.68	0.80	0.38	0.53	0.86	0.89	0.00																		
Blackstone (BLA)	0.60	0.58	0.66	0.61	0.55	0.57	0.51	0.70	0.62	0.64	0.65	0.85	0.00																	
Ran thembili (RT)	0.58	0.54	0.63	0.66	0.43	0.52	0.47	0.67	0.63	0.61	0.40	0.72	0.50	0.00																
Bodiri (BOD)	0.53	0.72	0.65	0.64	0.48	0.49	0.52	0.45	0.54	0.68	0.51	0.64	0.61	0.62	0.00															
Dwarf Brown (DB)	0.41	0.77	0.90	0.58	0.60	0.63	0.76	0.38	0.47	0.85	0.78	0.38	0.85	0.67	0.62	0.00														
Kamandala (KAM)	0.85	0.68	0.38	0.70	0.46	0.56	0.36	0.83	0.84	0.45	0.38	0.89	0.67	0.54	0.56	0.89	0.00													
Gon thembili (GT)	0.70	0.68	0.48	0.57	0.54	0.66	0.32	0.83	0.62	0.35	0.33	0.89	0.63	0.59	0.61	0.94	0.47	0.00												
Pora pol (PP)	0.84	0.86	0.49	0.72	0.56	0.66	0.27	0.94	0.71	0.41	0.28	0.84	0.80	0.56	0.70	0.94	0.43	0.20	0.00											
Nawasi (NAW)	0.80	0.63	0.48	0.70	0.29	0.46	0.32	0.84	0.78	0.45	0.23	1.00	0.58	0.54	0.56	0.83	0.37	0.37	0.31	0.00										
Dwarf red (DR)	0.50	0.71	0.95	0.62	0.68	0.68	0.90	0.42	0.58	1.00	0.89	0.29	0.69	0.71	0.83	0.23	0.84	0.84	1.00	0.84	0.00									
Dwarf yeolw (DY)	0.62	0.83	0.90	0.67	0.73	0.74	0.90	0.44	0.85	1.00	0.89	0.38	0.70	0.78	0.70	0.31	0.89	0.84	1.00	0.84	0.23	0.00								
Mirishena dw (MID)	0.46	0.71	0.90	0.58	0.60	0.63	0.85	0.38	0.47	1.00	0.89	0.38	0.85	0.67	0.52	0.19	0.89	0.94	1.00	0.84	0.16	0.31	0.00							
Rathran thembili (RT)	0.68	0.89	0.85	0.77	0.78	0.79	0.76	0.38	0.65	0.84	0.78	0.56	0.78	0.78	0.62	0.38	0.77	0.83	0.83	0.83	0.35	0.38	0.38	0.00						
Dwarf green (DG)	0.46	0.77	0.95	0.53	0.69	0.68	0.85	0.38	0.53	1.00	0.89	0.38	0.60	0.67	0.58	0.19	0.94	0.94	1.00	0.84	0.16	0.31	0.13	0.25	0.00					
Kasagala (KAS)	0.77	0.86	0.80	0.80	0.37	0.45	0.40	0.89	0.70	0.49	0.33	0.79	0.62	0.57	0.59	0.74	0.51	0.41	0.42	0.32	0.84	0.79	0.84	0.74	0.84	0.00				
Deberayaya (DEB)	0.80	0.59	0.49	0.74	0.43	0.48	0.33	0.89	0.74	0.46	0.20	0.89	0.41	0.45	0.62	0.78	0.44	0.38	0.28	0.23	0.89	0.83	0.89	0.78	0.89	0.24	0.00			
Clovis (CLO)	0.65	0.64	0.75	0.65	0.59	0.57	0.84	0.61	0.72	1.00	0.82	0.61	0.71	0.69	0.67	0.66	0.73	0.86	0.90	0.86	0.56	0.51	0.51	0.71	0.56	0.70	0.82	0.00		
Cameroon RD (CRD)	0.41	0.66	0.90	0.49	0.60	0.63	0.80	0.25	0.47	1.00	0.83	0.38	0.80	0.61	0.52	0.19	0.89	0.89	0.94	0.89	0.16	0.31	0.13	0.38	0.13	0.89	0.83	0.51	0.00	
Damana (DAM)	0.75	0.74	0.52	0.70	0.46	0.61	0.36	0.94	0.84	0.56	0.33	1.00	0.63	0.64	0.61	0.89	0.53	0.42	0.43	0.26	1.00	1.00	1.00	0.89	1.00	0.41	0.33	0.86	0.94	0.00

home garden in Colombo, obtained by the landowner as a gift from a traveler who brought the seedling from the Philippines and this palm produces large nuts similar to that of San Ramon. The earlier classification of king coconut (*aurantiaca*) forms as an intermediate variety between tall and dwarf varieties becomes somewhat controversial by the present findings. All forms of king coconut groups fall well within dwarf coconut forms. Even most of the morphological (slender trunk) and reproductive characters (autogamy and profuse bearing capacity) of king coconut also places it more towards a dwarf than a typical Sri Lanka Tall. Similar observations have also been reported by Perera *et al.* (1998; 2000) through variation detected by amplified fragment length polymorphisms (AFLPs) and SSRs respectively. Further, the current taxonomic status of Bodiri as a Sri Lanka tall is also challenged by this study. This variety also possesses many dwarf specific characters such as profuse bearing ability and a certain degree of autogamy.

The second group consisted entirely of all tall coconut collections and few unknown tall forms. This major group comprised two sub groups; one only with Sri Lanka tall accessions the other with tall coconuts but not typical of Sri Lankan Tall except *Ran Thembili*, which earlier described as a phenotypic deviants of the Sri Lankan Tall. The close clustering of all Sri Lanka tall ecotypes in the first sub cluster within this main group 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 and height and elongated average sized 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 markers. The accessions in the second sub cluster were *Ran Thembili*, Blackstone, Dickwella, Indian and San Ramon-*Ran Thembili*. Everard *et al.* (2000) have described that Dickwella and Blackstone are phenotypically similar producing nuts, which are big and round to oval in shape somewhat similar to San Ramon nuts in their appearance. Indian is a collection obtained from a single palm from a home garden in Gampaha. It has been named as "Indian" on the information given by the landowner that it was given to him by a person claiming that it was of Indian origin. This coconut is more similar to San Ramon than Sri Lankan or Indian Tall coconut. San Ramon-*Ran Thembili* is also a collection from a single palm from the same home garden in Gampaha which was named as San Ramon-*Ran Thembili* because the nuts of this palm was very much similar to that of San Ramon but with pink coloured ring in the mesocarp close to carpels in the young fruit, which was characteristic of the *Ran Thembili* (Sri Lanka Tall form) and *Rathran Thembili* (king coconut form). It is interesting to note that both San Ramon-*Ran Thembili* and *Ran Thembili* found in the same cluster deviating from their expected positions in the current taxonomic status that former should group with Clovis while latter with Sri Lanka Tall. Since Indian and San Ramon-*Ran Thembili* come from just two seeds raised it is very likely they have resulted from open pollination between a parent palm probably of exotic origin. However, the pollen parent is likely to be a Sri Lankan Tall making their appearance justifiable in this cluster. All the germplasm accessions of Sri Lanka Tall have a similar genome indicating that they share a narrow genetic base. The dwarf coconuts in Sri Lanka share a common genome, which is more close to the genome of coconuts in the Pacific and South East Asia, where the coconut was believed to have originated.

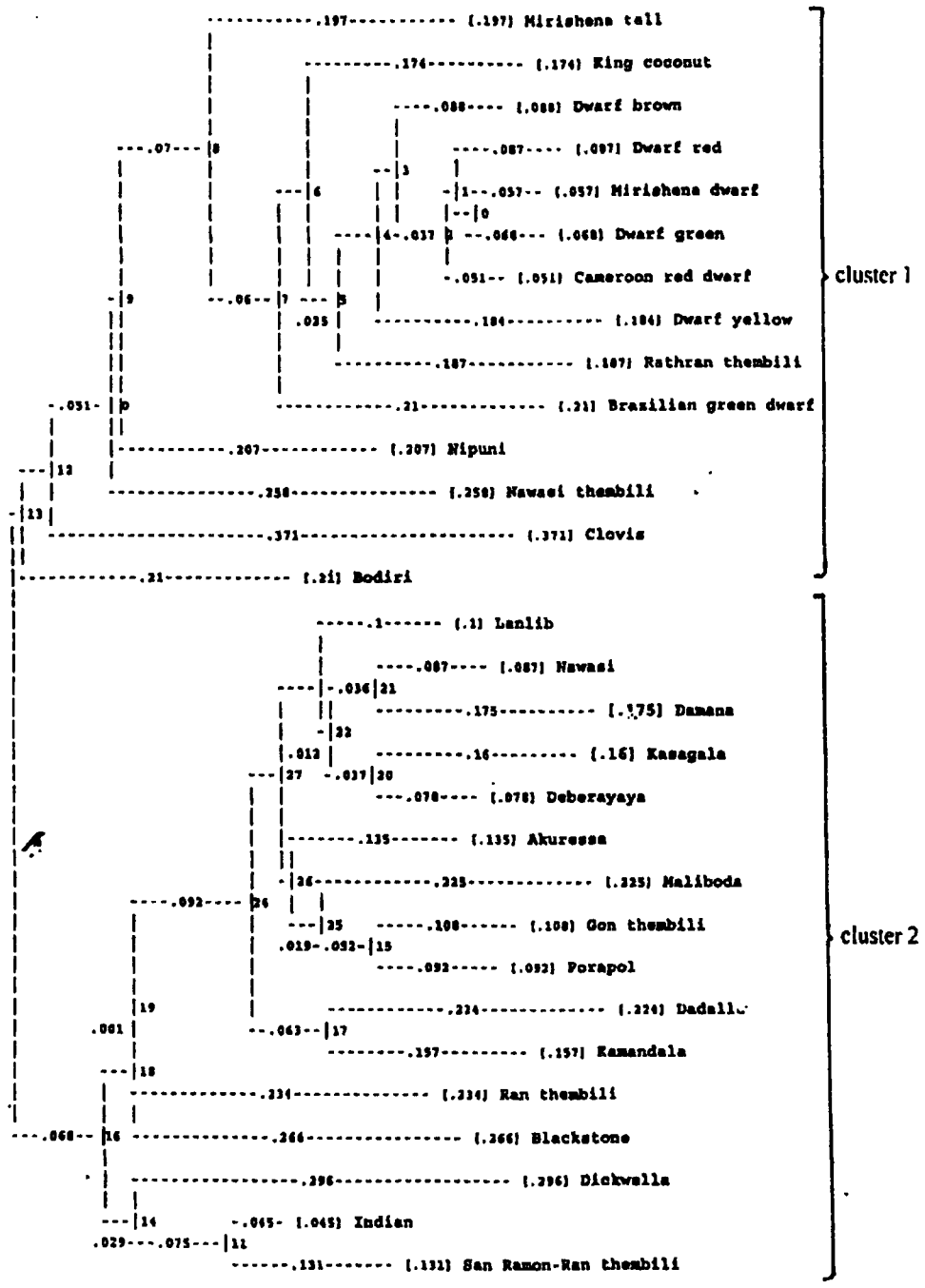

Figure 1: Dendrogram of 30 *ex-situ* conserved coconut germplasm in Sri Lanka based on Nei and Li genetic distances calculated on microsatellite band sharing ratio.

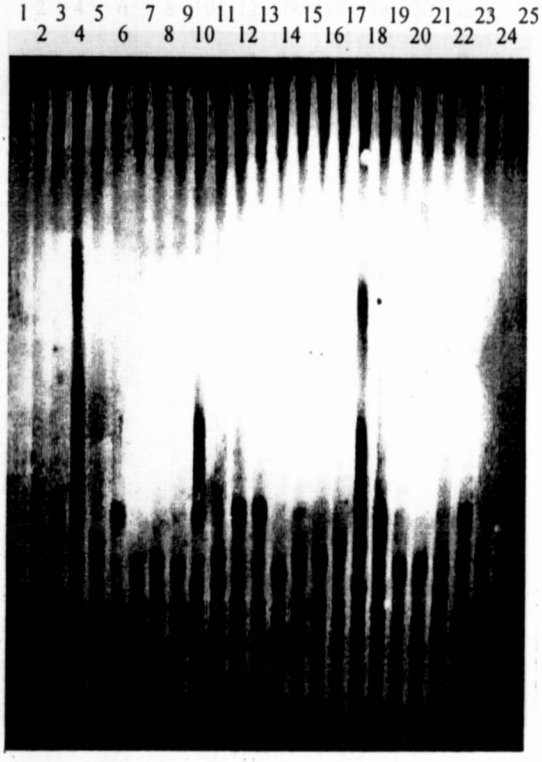

Fig. 2. **Microsatellite polymorphisms in *ex situ* conserved coconut germplasm accessions in Sri Lanka detected by the primer CNZ21**. Lanes 1-25 are DNA sources Mirishena Tall (MHT), Wilhelmina (WHM), Dickwella (DIK), Yodakandiya (YOK), Dadalla (DAD), Iranawila (IRW), Nipuni (NIP), Indian (IND), San Ramon - Ran Thembili (SRT), Tall Badirippuwa Estate (TBE), Akuresasa (AKU), Green Dwarf Kundasale (GDK), King Coconut (KCT), Nawasi Thembili (NWT), Maliboda (MLB), Margaret (MGT), Lanlib (LLB), Wanathawillu (WAW), Brazilian Green Dwarf (BGD), Blackstone (BLS), Ran Thembili (RAT), Bodiri (BOD), Dwarf Brown (DWB), Kamandala (KMD) and Gon Thembili (GNT).

The overall results suggest diverse groups as dwarf, tall coconut forms typical of Philippine and Sri Lanka tall as prospective groups for testing crosses for hybrid vigor. It is interesting to note that these three categories have already being tested and proved successful by the breeders of the Coconut Research Institute resulting in release of cultivars, Dwarf green/yellow x Sri Lanka Tall (CRIC65), Sri Lanka Tall x Clovis/San Ramon (CRISL98). The narrow genetic base of the Sri Lanka Tall coconut population suggests giving emphasis on collecting and conservation of more ecotypes of Sri Lanka Tall is futile for future breeding purposes. Therefore, it is imperative to enrich the diversity of coconut palm population by introduction of more diverse exotic groups for testing more prospective hybrids.

CONCLUSIONS

The 17 SSR primers have proven adequate to elucidate the genetical structure of the coconut palm population in Sri Lanka. Sri Lanka's coconut palm population is made of Sri Lanka Tall which probably comes from a single population either from India or Africa. The other available coconut diversity is mainly the dwarf coconut palms and a few Philippine type tall coconut palms. Since these diverse groups of coconut has already been successfully utilized in breeding it is important to enrich the coconut population in Sri Lanka by introduction of more exotic forms of coconut rather than attempting to make use of the genetic variation available within these groups.

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