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Evaluation of Genetic Variation of Exotic Coconuts (*Cocos nucifera* L.) using SSR Markers

F.S. SUHAIR[1], S.A.C.N. PERERA[2] and K. VIVEHANANTHAN[1]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP).*

[2]*Genetics and Plant Breeding Division, Coconut Research Institute of Sri Lanka, Lunuwila.*

ABSTRACT

Coconut (*Cocos nucifera* L.) is an important plantation crop in Sri Lanka. Therefore crop improvement through breeding has become a priority research area in this crop. Breeding coconut is a challenging task due to limitations inherent to the palm and molecular markers can play a vital role by assisting in overcoming some of these limitations. The objective of the current research was to study the genetic variation of exotic coconuts imported to Sri Lanka and conserved in field gene banks. Genetic variations were detected at 5 microsatellite loci CNZ06, CNZ04, CAC65, CAC02, and CNZ44. DNA was extracted from 43 leaf samples belonging to both Sri Lankan and imported varieties from Papua New Guinea, Ivory Coast and India. Extracted genomic DNA was PCR amplified, electrophoresed in polyacrylamide gels and bands were visualized after silver staining and data were analyzed in Powermarker software. The results revealed that exotic tall coconut varieties are more heterogeneous than local tall varieties. The study further revealed the potential for using exotic varieties for breeding purposes to extract hybrid vigour due to their genetic variability compared with the local varieties.

KEYWORDS: Coconut, Exotic varieties, SSR markers

INTRODUCTION

Coconut (*Cocos nucifera* L.) is an extensively grown and used nut in the world. It plays a significant role in the economical, cultural and social life of people in coconut growing countries.

Coconut has dispersed from its putative origin, to many different parts of the world including central and south America, East and West Africa, South East Asia, East Asia and Pacific Islands. Among coconut growing countries Philippines, Indonesia, India, Papua New Guinea, Mexico Malaysia, Vietnam, Sri Lanka, Mozambique, Tanzania and Ivory Coast are note worthy. Although today Sri Lanka is the 4th largest coconut producing country in the world, the percentage contribution to the worldwide production is only 5%. But the coconut sector plays an important role in contributing to the national economy ensuring food and nutrition security to a larger segment of the population. It accounts for about 1.7% of the Gross Domestic Production and contributes 2.74% of foreign exchange earning annually (Plantation Sector Statistical Pocket Book, 2007).

All coconuts in general fall into two categories, tall (typica) varieties and dwarf (nana). Tall varieties are predominantly out breeding in their reproduction behavior and hence highly heterozygous in nature. They are hardy palms with a tall structure (average height 18 m) and able to withstand harsh climatic conditions such as drought.

Dwarf coconuts in contrast are predominately inbreeding and more homozygous in nature. They are more soft and less able to withstand harsh climatic conditions and more prone to pest and diseases.

The molecular makers can play a major role in assisting coconut breeding mainly *via* germplasm characterization, genome mapping and marker assisted selection (MAS). Molecular marker systems, RAPDs, AFLPs, SSR and DArT have so far been used in related research to coconut breeding and improvement (Everard, 1996; Perera *et al.*, 1998; Perera *et al.* 2001; Perera, 2008).

Among other molecular markers, SSR markers were the most extensively used markers in coconut breeding and currently there are several hundreds of coconut specific SSR primers identified.

Some countries implement breeding programmes using limited number of varieties which may not be suitable or adequate enough to effectively achieve their breeding objectives. Several countries attempted and succeeded in coconut germplasm exchange programs. For example, Sri Lanka exchanged germplasm with India, Papua New Guinea and Ivory Coast. These imported material need to be characterized to identify genetic variation among them for them to be effectively used in the coconut breeding programme in the country. Therefore the objective of the current study is to characterize the exotic imported germplasm using SSR markers to identify their genetic variation at microsatellite marker loci.

MATERIALS AND METHODS

The study was conducted at the Genetics and Plant Breeding Division of the Coconut Research Institute, Lunuwila, Sri Lanka during the period from January to April 2013.

Exotic varieties have been imported from Papua New Guinea, Ivory Coast and India (Table 1) during 2002-2004.

Table1. Country, Variety/Form and the sample order

Country	Variety/Form	Sample order
Sri Lanka	Green dwarf (SLGD)	1
	Red dwarf (SLRD)	2
	Yellow dwarf (SLYD)	3
	Brown dwarf (SLBD)	4
Papua New Guinea	Malayan red dwarf (MRD)	5
	Malayan yellow dwarf (MYD	6-7
Ivory Coast	Niulekha green dwarf (NGD)	8-9
	Cartigan green dwarf (CGD)	10
	Tagnanan tall (TGD)	11
Papua New Guinea	PNG Brown dwarf (PBD)	12
India	Banawali Round (BGR)	13-14
Sri Lanka	King coconut (KC)	15
	Sri Lankan tall (SLT)	16-17
	San Rammon (SR)	18
Papua New Guinea	Kar Kar Tall (KKT)	19-20
India	Andaman Tall (AO)	21-22
	Laccadive Ordinary (LO)	23-24
Papua New Guinea	Rannel Island tall (RT)	25-26
India	West coast tall (WCT)	27-28
Papua New Guinea	Markem valley tall (MKV)	29-30
	Gazelle Peninsula tall (GT)	31-32
Ivory Coast	West African tall (WAT)	33-34
	Vanuatu tall (VT)	35-36
	Tagnanan tall (TGT)	37-38
	Tanga tall (TNT)	39-40
	Tahitian Tall (TAT)	41-42
	Polynesia tall (PT)	43

DNA Extraction and Quantification

Genomic DNA was extracted from the leaves of 43 palms according to the modified CTAB (4%) procedure described by Rogers and Bendich (1985), Doyle and Doyle (1987).

Two microlitres of DNA was mixed with 4 µl of (10X) gel loading buffer (mixture of 60% Glycerol, 0.025% of Bromophenol blue, 0.025% of Xylene Cynol) and 4 µl of sterilized distilled water and the mixture was loaded on a 0.8% Agarose gel containing Ethidium bromide for electrophoresis.

Gel was run at 85 V using 0.5 X TBE buffer until the dark blue Bromophenol blue dye has migrated to two-thirds from the length of the gel. Bands were observed under UV light for detection of extracted DNA to determine the quality and quantity. Quantified DNA was diluted to obtain a DNA concentration of 10 pm/µl before PCR amplification.

PCR Amplification

PCR amplification was performed for five microsatellite loci (Table 2) in 43 DNA samples. PCR programme consisted of an initial denaturation (94°C for 4 min), 35 cycles each consisting of 30 sec 94°C, 30 sec annealing temperature depending on the primer used (Table 1) and 1 min extension (72°C). At the end of the final cycle an extension period of 5 min at 72°C was included. Final holding temperature was 4°C.

Detection of PCR Product

Amplified PCR products were detected by polyacrylamide gel electrophoresis, using a BIORAD sequi-gene GT apparatus. The gel mix consisted with 100 ml of acrylamide\bis acrylamide, 600 µl of 10% Ammonium persulfate (APS) and 60 µl of TEMED. Gel running buffer was 1X TBE. Three microlitres of loading buffer was added to the 10 µl PCR Bromophenol blue dye has migrated to two-thirds from the length of the gel. Bands were observed under UV light for detection of extracted DNA to determine the quality and quantity. Quantified DNA was diluted to obtain a DNA concentration of 10 pm/µl before PCR Amplification and 4.5 µl of the mix was loaded into each well of the gel and 100 bp ladder at the 2 ends of the gel. Staining of the gel after running was done in 3 steps.

Firstly the gel was fixed in a solution containing 10 ml of glacial acetic acid and 2 L of sterile distilled water for 20 min over night. The gel was washed with distilled water for several times. It was then dipped in the staining solution containing 3.2 g of silver nitrate dissolved in 2 L of distilled water. Staining was done for 20 min by shaking. The gel was washed once with distilled water and developed by dipping in a solution containing 12 g of NaOH, 10 ml of formaldehyde and 2 L of distilled water in a separate tray.

Bands were visually detected and the patterns were scored.

Data Analysis

The data were analyzed in Powermarker software to derive allele and gene diversity and heterozygosity.

Table 2. Forward (F) and Reverse (R) SSR primer sequences and annealing temperatures

Primer	Sequence	Annealing Temperature
CNZ 44	F 5'- CAT CAG TTC CAC TCT CAT TTC-3' R 5'- CAA CAA AAG ACA TAG GTG GTC-3'	53°C
CNZ 06	F 5'- ATA CTC ATC ATC ATA CGA CGC-3' R 5'- CTC CCA CAA AAT CAT GTT ATT-3'	53°C
CNZ 04	F 5'- TAT ATG GGA TGC TTT AGT GGA-3' R 5'- CAA ATC GAC AGA CAT CCT AAA-3'	53°C
CAC 02	F 5'- AGC TTT TTC ATT GCT GGA AT-3' R 5'- CCC CTC CAA TAC ATT TTT CC-3'	53°C
CAC 65	F 5'- GAA AAG GAT GTA ATA AGC TGG-3' R 5'- TTT GTC CCC AAA TAT AGG TAG-3'	53°C

RESULTS AND DISCUSSION

DNA Extraction

The method for isolation of DNA from young coconut leaves by modified CTAB (4%) procedure was observed as appropriate for extracting DNA of the population. Sufficient quantities (67 µg/g of leaf tissue) of DNA were yielded with adequate quality for use in the SSR-PCR.

Detection by SSR markers

Visual observation of gel images (figure 1) and genotypic scores (Table 3) indicate the genetic variations among varieties/forms.

According to the analysis, number of alleles per maker ranged from 04 in CNZ06 to 10in CAC02 (Table 3).

Figure 1. Polyacrylamide gel image for primer CAC65. Lane 1-43: sample order (Table 2) Lane L: 100 bp ladder

Table 3 Summary statistics of molecular characterization

Maker	Number of observations	Allele number	Gene diversity	Heterozygosity
CNZ06	41	4	0.65	0.17
CNZ04	39	8	0.68	0.38
CAC65	40	8	0.63	0.40
CAC02	38	10	0.87	0.60
CNZ44	40	6	0.64	0.45
Mean	39.6	7.2	0.69	0.40

All the markers were polymorphic. The highest number of alleles were scored in CAC02 which can be taken as the most informative and polymorphic marker.

Mean allele number of all five markers, CNZ06, CNZ04, CAC65, CAC02, and CNZ44 is 7.2 (Table 3) indicating a higher level of allelic diversity in the population. When comparing the gene diversity CAC02 shows a higher number of diversity. Also CAC02 is the most heterozygous maker which gives heterozygosity of 0.6053 (Table 3)

When comparing the heterozygosity percentages, dwarf varieties showed comparatively low percentages than the tall varieties (Table 4) , which confirms that tall varieties are more heterogeneous due to their cross pollinating breeding behavior resulting in several allelic combinations within a population of tall coconuts.

Table 4 Heterozygosity percentages

	CNZ06	CNZ04	CAC65	CAC02	CNZ44
Dwarf	13%	13%	13%	20%	13%
Tall	17%	53%	50%	71%	50%
SLT	0%	0%	0%	0%	0%

All Sri Lankan tall varieties showed a heterozygosity percentage of 0%. So contribution to the heterozygosity percentage in this population is totally from the exotic tall coconut varieties. It reveals that exotic tall coconut varieties are more heterozygous and as a result more heterogeneous than local tall varieties.

CONCLUSIONS

The study reveals a higher genetic variation and a rich allelic diversity in the exotic coconut varieties imported to Sri Lanka. Therefore there is a very high potential to extract hybrid vigour by the use of these exotic coconut germplasm in the local coconut breeding programmes.

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