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Inter Simple Sequence Repeat (ISSR) markers for identification of germplasm and diversity analysis in coconut (*Cocos nucifera* L.)

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ABSTRACT

Inter Simple Sequence Repeat (ISSR) is a powerful molecular marker technique used for variety of purposes like genetic diversity analysis, phylogeny information, germplasm identification, gene tagging etc. In the present paper, 33 coconut (*Cocos nucifera* L.) germplasm representing different geographical regions have been fingerprinted with highly polymorphic ISSR markers. Ten polymorphic primers targeting the microsatellite regions were used to amplify the coconut germplasms. Ten primer produced 104 polymorphic markers with 87 percent polymorphism. Cluster analysis was carried out among 33 accessions by UPGMA using the software POPGENE ver.1.32. The dendrogram revealed five clusters. Based on the fingerprint data germplasm specific markers were identified using the primers UBC 810, UBC 915, UBC 823, UBC 824 and UBC 855. The marker UBC 815_{1673} was specific to Panama Tall (PNT) and Saint Vincent Tall (STVT). The marker UBC 854_{573} was specific to Nuwehnug Tall (NWHT). ISSR markers were proved to be useful for generating fingerprints and for deriving genetic relationships in coconut.

Keywords: Coconut, ISSR markers, germplasm, specific markers

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is one of the major perennial oil crops of tropics providing the basis for food and industrial products in many developing countries. So far, germplasm accessions have been characterized and identified using morphological and nut characteristic such as plant stature as tall/dwarf, crown shape, leaf characters, nut shape, nut color etc. These characteristics are still continued to be used in the germplasm bank at Central Plantation crops Research Institute (CPCRI), India. But, the task of unambiguously identifying the new accessions will be difficult by the use of conventional characteristics alone. It is apparent that use of molecular markers would solve the problem of providing unique DNA profiles for the germplasms, with aid of morphological characteristics.

Molecular markers like RFLPs (Lebrun *et al.*, 1998), RAPD (Everard, 1999, Upadhyay *et al.*, 2004), AFLP (Perera *et al.*, 1998), SSR (Perera *et al.*, 2000, 2003. Rajesh *et al.*, 2008) and ISSR (Manimekalai, 2005) have been reported in coconut for genetic diversity estimation. Cultivar specific markers from retrotransposon-based insertional polymorphism (RBIP) based on the long terminal repeat (LTR) sequences have been developed for Japnese Pear (Kim *et al.,* 2012) and for the analysis of diversity (Kalendar *et al.*, 2011). The cost involved in the development of SSR markers necessitates the use of Inter Simple Sequence Repeat (ISSR) markers. ISSR PCR is a technique that overcomes the problems like low reproducibility of RAPD, high cost of AFLP, and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Goodwin *et al.*, 1997). ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10 mers) which permit the subsequent use of high annealing temperature (45-60 °C) leading to higher stringency. Because of high polymorphism and reproducibility, it seemed likely that this maker would be useful for germplasm identification in coconut. The objective of the work reported here was to assess the ability of ISSR to distinguish the coconut accessions and to discern genetic relationships among a set of coconut germplasm.

MATERIALS AND METHODS

DNA was extracted from 2g of fresh leaf material from 132 individuals of 33 germplasm (4 individuals per accession) using Plant DNA extraction kit (In vitrogen) as per the manufacturer's instructions (Table 1). Amplification reactions were carried out as per the standardized protocol (Manimekalai, 2005). ISSR primer sequences and primers were supplied by University of British Columbia, Canada. Ten highly polymorphic primers were used to amplify the DNA. These primers were selected based on preliminary primer screening of 40 ISSR primers and then selected highly polymorphic 10 primers. Amplifications were performed in a Thermo Cycler (MJ Research Inc.) programmed for an initial denaturation at 94 °C for 5 min., 40 cycles of 1 min., denaturation at 94 °C, 1 min., annealing at 50 °C and 2 min. extension at 72 °C and a final extension of 7 min. at 72 °C. The PCR products were electrophoresed in 1.80 % agarose gels.

Only the clear, unambiguous and reproducible bands were considered for scoring. Each band was considered to represent 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 and 100 bp DNA ladder or lambda DNA Eco RI / Hind III double digest (MBI Fermentas, Lithuania). Binary data were analyzed using the software POPGENE version 1.32 (Yeh and Boyle, 1999). The dendrogram was constructed using UPGMA based on Nei's unbiased genetic distance in the software Phylip 3.5. The markers produced by the individual primer were labeled by suffixing the molecular weight of the respective band.

RESULTS AND DISCUSSION

Ten highly polymorphic ISSR primers detected a total of 120 markers across 33 coconut germplasms, out of which 104 were polymorphic. The number of markers per primer varied from nine (UBC 854) to 17 (UBC 854), with a mean of 12 markers per primer. The number of polymorphic markers for each primer varied from seven (UBC 835) to 15 (UBC 854 and UBC 855) with a mean of 10.4. The product size ranged from 206 bp (UBC 834) to 2618 bp (UBC 815) (Table 2). Fingerprint pattern produced by the primer UBC 889 (Fig. 2).

ISSR primers produced specific markers for germplasm. The primer UBC 810 produced three markers (Table 3).

Table 1: Details of coconut germplasm accessions

No.	Accession	Code	Geographic region
1.	Kong Thienyong Tall	KTYT	Southeast Asia
2.	Straight Settlement Green Tall	SSGT	Southeast Asia
3.	Straight Settlement Apricot Tall	SSAT	Southeast Asia
4.	Philippines Kalambahim Tall	PKBT	Southeast Asia
5.	Laguna Tall	LAGT	Southeast Asia
6.	Philippines Palawan Tall	PPWT	Southeast Asia
7.	Philippines Dalig Tall	PDLT	Southeast Asia
8.	San Roman Tall	SNRT	Southeast Asia
9.	Markham Valley Tall	MVT	South Pacific
10.	Nufella Tall	NUFT	South Pacific
11.	Nugili Tall	NUGT	South Pacific
12.	Nuwallis Tall	NUWT	South Pacific
13.	Nu Quamen Tall	NUQT	South Pacific
14.	Kupien Tall	NHKT	South Pacific
15.	Nuwehnug Tall	NWHT	South Pacific
16.	Lifou Tall	LFT	South Pacific
17.	British Solomon Island Tall	BSIT	South Pacific
18.	Jamaica Tall	JMT	Atlantic
19.	Saint Vincent Tall	STVT	Atlantic
20.	Panama Tall	PNT	America
21.	Nigerian Tall	NIT	Africa
22.	Kaithathali Tall	KAIT	South Asia
23.	Indian Spicata	WCT01	South Asia
24.	Indian East Coast Tall	ECT	South Asia
25.	Verrikobbari Tall	VKBT	South Asia
26.	Nadora Tall	NDRT	South Asia
27.	Nicobar Tall	NICT01	South Asia
28.	Hazari Tall	HZT	South Asia
29.	Navassi Tall	NAVT	South Asia
30.	Niuleka Dwarf	NLAD	South Pacific
31.	King coconut	RTB04	South Asia
32.	Laccadive Dwarf	LCOD	South Asia
33.	Chowghat Orange Dwarf	COD	South Asia

The marker UBC 855$_{2155}$ was unique to dwarf coconuts studied here and two of the South Pacific coconut populations, Nufella Tall (NUFT) and Nuwallis Tall (NUWT). The marker UBC 815$_{1673}$ was specific to Panama Tall (PNT) and Saint Vincent Tall (STVT). UBC 889$_{914}$ was specific to Philippines Palawan Tall (PPWT), San Ramon Tall (SNRT), Nuwallis Tall (NUWT), Saint Vincent Tall (STVT), of Nicobar Tall (NICT 01), Kaitha4thali Tall (KAIT) and Hazari Tall (HZT). This is the first report of use of ISSR markers in germplasm identification in coconut, however they are available in other crops. In cashew ISSR fingerprints have been developed for the germplasm identification (Archak *et al.*, 2003)

Dendrogram was constructed based on average genetic distance among 33 coconut germplasming (Fig. 1) by UPGMA method. The dendrogram revealed five clusters. Cluster I consisted of germplasm belonging to the South East Asia (KTYT, SSGT, SSAT, PKBT, LAGT, PPWT, SNRT) and South Pacific region (MVT, NUWT, NUQT, LFT) . Dwarf and intermediate population (LCOD, NLAD, RTB 04) and Indian Spicata (WCT 01) also clustered within cluster I. Indian Spicata (WCT 01) which is characterized by a single spike, without any spikelets that bears many female flowers is generally considered a variant of West Coast Tall (WCT) in India. It showed clear proximity to South East Asia coconut in this study. Previous reports (Manimekalai and Nagarajan, 2006) also suggested the clustering of Indian Spicata coconuts with South East Asian accessions. The dwarf coconuts clustering with the South East Asia coconuts are in agreement with the earlier report

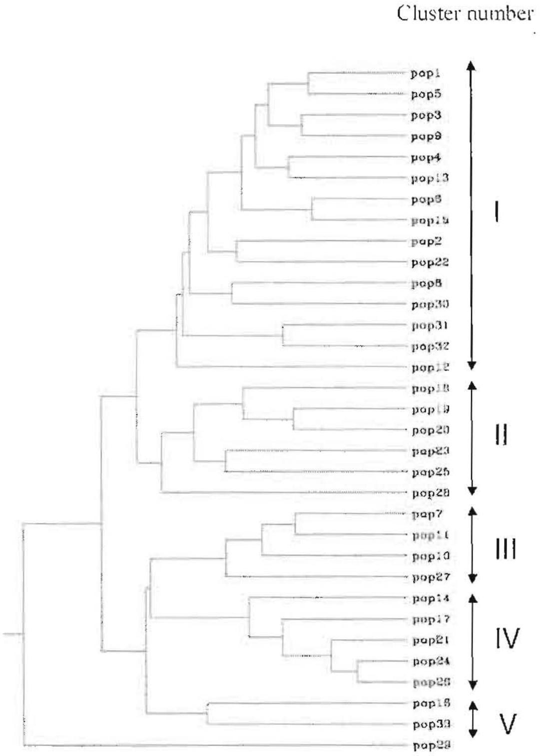
Table 2: Details of primers used and markers produced in coconut germplasms

S.No.	Primer	Sequence	Total markers (No.)	Polymor phic markers (No.)	Product size (bp)
1.	UBC815	CTCTCTCTCTCTCTCTG	13	13	2618-554
2.	UBC834	AGAGAGAGAGAGAGAGYT	12	10	1252-206
3.	UBC841	GAGAGAGAGAGAGAGAYC	10	8	2316-698
4.	UBC810	GAGAGAGAGAGAGAGAT	9	8	2443-879
5.	UBC824	TCTCTCTCTCTCTCTCG	10	9	2455-506
6.	UBC835	AGAGAGAGAGAGAGAGYC	10	7	2375-514
7.	UBC854	TCTCTCTCTCTCTCTCRG	17	15	1545-299
8.	UBC855	ACACACACACACACACYT	15	15	2459-606
9.	UBC889	DBDACACACACACACAC	14	10	2069-290
10.	UBC823	TCTCTCTCTCTCTCTCC	10	9	2287-877
	Total		120	104	
	Mean		12.0	10.4	

Y= (C, T)
D= (A, G, T)
R= (A, G)
B=(C,G,T) ie not A

Table 3: List of ISSR markers identified for the coconut germplasms

S. No.	ISSR marker	Germplasms identified
1.	UBC 889 $_{914}$	Philippines Palawan Tall (PPWT), San Roman Tall (SNRT), Nuwallis Tall (NUWT), Saint Vincent Tall (STVT), Kaithathali Tall (KAIT), Nicobar Tall (NICT 01) and Hazari Tall (HZT)
2.	UBC 810 $_{1543}$	Hazari Tall (HZT), Niu Leka Dwarf (NLAD) and King coconut (RTB 04)
3.	UBC 810 $_{879}$	Kupien Tall (NHKT)
4.	UBC 810 $_{674}$	Straight Settlement Apricot Tall (SSAT) and Hazari Tall (HZT)
5.	UBC 815 $_{1673}$	Saint Vincent Tall (STVT) and Panama Tall (PNT)
6.	UBC 823 $_{1081}$	Straight Settlement Apricot (SSAT), Nuwallis Tall (NUWT) and Navassi Tall (NAVT)
7.	UBC 824 $_{1008}$	Jamaica Tall (JMT), Panama Tall (PNT), Nigerian Tall (NIT) and Nadora Tall (NDRT)
8.	UBC 824 $_{939}$	Hazari Tall (HZT), San Roman Tall (SNRT), Markham Valley Tall (MVT), Philippines Kalambahim Tall (PKBT), Nu Quamen Tall (NUQT), Jamaica Tall (JMT), Panama Tall (PNT), Saint Vincent Tall (STVT), Nigerian Tall (NIT) and Chowghat Orange Dwarf (COD)
9.	UBC 854 $_{952}$	Straight Settlement Apricot Tall (SSAT)
10.	UBC 854 $_{573}$	Nuwehnug Tall (NWHT)
11.	UBC 855 $_{2155}$	Nufella Tall (NUFT), Nuwallis Tall (NUWT), King coconut (RTB 04), Laccadive Dwarf (LCOD) and Chowghat Orange Dwarf (COD)



POP1=KTYT; POP2=SSGT; POP3=SSAT; POP4=PKBT; POP5=LAGT; POP6=PPWT; POP7=PDLT;POP8=SNRT; POP9=MVT; POP10=NUFT; POP11=NUGT; POP12=NUWT; POP13=NUQT; POP14=NHKT; POP15=LFT; POP16=BSIT; POP17=JMT; POP18=STVT; POP19=PNT POP 20=NIT; POP21=KAIT; POP22=WCT01; POP23=ECT; POP24=VKBT; POP25=NDRT; POP26=NICT01; POP27=NWHT POP28=HZT; POP29=NAVT POP30=NLAD; POP31=RTB04; POP32=LCOD; POP33=COD)

Fig. 1: Dendrogram of 33 coconut germplasm based on 120 ISSR markers constructed using UPGMA based on Nei's unbiased genetic distance in the software Phylip 3.5

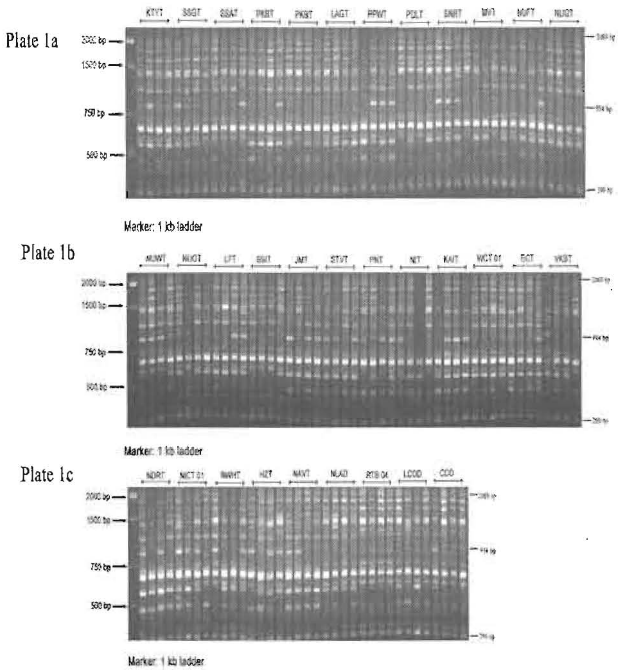


Fig. 2: UBC 889 ISSR profile of coconut accessions showing specific markers

(Perera *et al.*, 2003). Cluster II comprised accessions belonging to South Asian (ECT, HZT, NDRT), Atlantic and American populations (PNT, STVT) and African populations (NIT). Generally, a grouping of population based on the geographic origin was observed, except in Philippines Dalig Tall (PDLT), Kaithathali Tall (KAIT), Verrikobari Tall (VKBT), Nicobar Tall (NICT 01) and Indian Spicata (WCT 01). The clustering pattern of coconut population obtained here using ISSR markers is comparable with that of clusters produced by RAPD markers (Lebrun *et al.*, 1998). The accession Navassi tall (NAVT) occupied separate position in the dendrogram, even though this accession belongs to the South Asia region, It reveals that the NAVT has variability among South Asian accessions. Based on our study, ISSR markers are found to be useful in generating fingerprints for coconuts and to infer genetic relationships among the coconut accessions. Moreover, ISSR technique is robust compared to RAPD, technically less demanding than AFLP and less complex than SSR. With the less infrastructure facility this technique could be performed. Hence, ISSR markers have potential in genotypic studies in coconut. Barcode for the Palmae family species are being developed (Jeanson *et al.*, 2011) based on *matK, rbcL* and nrITS2 for species identification, however, for germplasm identification, highly polymorphic markers are useful than the barcodes.

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