



Development of a RAPD-derived SCAR marker associated with tall-type palm trait in coconut

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ABSTRACT

Coconut palms are classified into two major types, viz. 'talls' and 'dwarfs', based on plant stature. The selection of coconut seedlings in the nursery, presently relying solely on morphological markers, often results in selection of out-crossed seedlings. Hence, identification of molecular markers for distinguishing tall/dwarf character at an early stage of growth assumes importance. In the present investigation, a RAPD marker for tall-type palm trait was identified using a pooled DNA approach. Screening of tall and dwarf palm bulked DNA with 200 decamer primers revealed a primer OPA09, producing a unique band of around 260 bp exclusively in tall accessions. The primer was used for screening and validation in individual tall and dwarf coconut accessions representing different geographic regions. The band was present in all the tall accessions, but absent in the dwarfs. Furthermore, sequence characterized amplified region (SCAR) primers were designed from the unique RAPD amplicon. The primers produced a specific 260-bp amplicon in tall accessions, but not in dwarf accessions. The SCAR marker was utilized in assessing the purity of hybrid seedlings of D × T (Dwarf × Tall) cross. The results pave the way for ensuring genuineness/quality of hybrid seedlings of coconut.

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1. Introduction

Coconut palm (*Cocos nucifera* L.), an important plantation crop grown traditionally for oil and fibre around the tropical regions of the world, is now transforming into a food, beverage and nutrition crop. Millions of people in 93 countries depend on coconut cultivation for their livelihood security. The fresh kernel of coconuts is used in a number of culinary preparations and snacks. The tender nuts provide a natural, hygienic, nutritive refreshing drink with a nutritious and delicious young kernel. The fresh palm cabbage, i.e. the growing bud, is served as an excellent table purpose salad and the roots are used in traditional Indian ayurvedic preparations for many ailments. Coconut palm is also highly valued as an ornamental in lawns, golf courses and gardens. Considering the array of uses for its parts, the coconut palm is termed as '*Kalpavriksha*' meaning 'Tree of Heaven' or 'Tree of Life'.

Coconut palms, based on the plant stature and earliness, are classified into two major type's viz. 'talls' (*C. nucifera* L. var. *typica*) and 'dwarfs' (*C. nucifera* L. var. *nana*). While the tall cultivars are predominantly cross-pollinated, have higher height increments over the years due to longer internodes and are late bearing; the dwarf ones are mainly self-pollinated, have shorter internodes

and are early bearing (Liyanage, 1949; Menon and Pandalai, 1958; Foale, 2005). These traits can be distinguished without ambiguity only after five to six years of planting. The variation in breeding habit between the tall and dwarf cultivars results in the tall cultivars possessing higher levels of genetic diversity (Rao et al., 2005) than most dwarf cultivars. The tall cultivars are mostly cultivated for fresh kernel and fibre whereas the dwarf cultivars are mostly preferred for their earliness, sweet tender nut water and dwarf plant stature. Also, dwarf populations are generally distinct and uniform with regard to their fruit morphology and colour (Foale, 2005). The dwarf accessions are postulated to have evolved through inbreeding among the tall accessions (Nambiar and Swaminathan, 1961) and as a result of human selection. Although many dwarf and tall accessions have extensively been utilized in several breeding programmes, the inheritance of the dwarf trait in coconut has not been given attention due to the fact that the dwarf accessions mostly breed true to type, while talls and hybrids segregate.

A number of hybrid varieties have been developed in India by crossing selected tall and dwarf accessions for exploitation of hybrid vigour for nut yield and tender nut qualities (Ratnambal, 2001). However, one of the major hurdles in achieving planting material production of the released hybrids is obtaining genuine hybrid seedlings owing to the inherent heterozygosity in most of the tall cultivars and some of the dwarf cultivars. The lack of homogeneity in different mother palm populations results in segregation

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among F₁ progenies. Identification of pure tall and dwarf progenies at nursery stage, therefore, is a difficult task.

Hybrid seed production in coconut is done through selection of mother palms based on morphological traits, artificial pollination/hybridization using pollen from selected male parents, followed by seed nut production and seedling selection (Arunachalam and Rajesh, 2008). The most common morphological traits used for seedling selection include days taken for germination, vigour of seedlings in terms of leaf production, wider collar girth during a specific duration and most importantly, on comparison of the petiole colour of the progeny with the female and male parent (Arunachalam and Rajesh, 2008). Seedling selection relying only on the morphological parameters such as petiole colour and vigour of growth often results in selection of undesirable outcrossed seedlings and also rejection of possible good hybrids. The problem is doubled if the selection is for breeder seed planting material for establishment of mother gardens for seed production in developed varieties where the establishment of impure types could result in wastage of time, resources and loss of desirable genetic purity. Therefore, an alternative technique that offers efficient, quick and reliable assessment of genetic purity at seedling stage will be of significant use in strengthening coconut breeding programmes. Though several attempts have been made in the past for identification of molecular markers associated with specific traits in a long duration crop like coconut, it has so far been elusive for the important traits such as plant type. With this background, the present investigation was conducted using selected tall and dwarf coconut accessions for identification of molecular markers linked to plant-type trait.

2. Materials and methods

2.1. Plant materials

The plant material used for RAPD (Random Amplified Polymorphic DNA) analysis consisted of tall and dwarf accessions (Table 1),

which represent different geographic regions and conserved in the field gene banks of Central Plantation Crops Research Institute, Kasaragod, India, which is also designated as National Active Germplasm Site for Coconut.

2.2. DNA isolation and pooling

Initially, a rapid method for isolating of high quality DNA from spindle leaves of coconut was developed. DNA was extracted from approximately 1 g of fresh young leaves, collected from field grown parental plants and crushed in liquid nitrogen. The crushed powder was added to a tube containing 10 ml pre-heated extraction buffer (100 mM Tris-HCl, 50 mM NaCl; pH 8.0). To the above, 1 ml of 10% SDS and 50 µl of β-mercaptoethanol were added. The mixture was incubated at 65 °C for 1 h with intermittent mixing. An equal volume of chloroform:isoamyl alcohol mixture (24:1, V/V) was added after incubation and homogenized by gentle inversion for 20 min and centrifuged at 10,000 rpm for 20 min at 4 °C. The clear aqueous phase containing the nucleic acid was transferred to a fresh tube and 2/3rd volume ice-cold isopropanol was added to precipitate the nucleic acids. It was kept at 4 °C for 30 min and DNA pool was collected in 1.5 ml micro tubes. The pooled DNA was washed thrice with 70% alcohol. The alcohol was discarded and DNA pellet was air-dried completely and DNA pellet dissolved in 0.5 ml TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). RNase (4 µl) was added to the tube and incubated at 37 °C for 1 h. After RNase treatment, the supernatant containing the DNA was extracted with equal volume of chloroform: isoamyl alcohol (24:1) twice. The purified DNA was precipitated by adding double the volume of ethanol. The precipitated DNA was air-dried and dissolved in 0.75 ml TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA).

Two genotypic DNA pools were composed: a tall plant type pool including five tall cultivars (one genotype each of West Coast Tall, Fiji Tall, West African Tall, Sakhigopal Tall and Surinam Tall) and a dwarf plant type pool including five dwarf cultivars (two genotypes

Table 1
List of coconut accessions used for the present study.

Sl. no.	Name of the accessions	Abbreviation	Origin
<i>Tall accessions</i>			
1.	West Coast Tall	WCT	Kerala (India)
2.	Annur Tall	ANRT	Kerala (India)
3.	Bedakam Tall	BDKT	Kerala (India)
4.	Kappadam Tall	KPDT	Kerala (India)
5.	Sakhigopal Tall	SKGT	Odisha (India)
6.	Gangapani Tall	GPNT	Andhra Pradesh (India)
7.	Ayiramkachi Tall	AYRT	Tamil Nadu (India)
8.	Kulasekharam Tall	KGT	Tamil Nadu (India)
9.	Laccadive Ordinary Tall	LCT	Lakshadweep Islands (India)
10.	Laccadive Micro Tall	LMT	Lakshadweep Islands (India)
11.	Laccadive Giant Tall	LCGT	Lakshadweep Islands (India)
12.	Tiptur Tall	TPT	Karnataka (India)
13.	Benawali Green Round Tall	BENT	Maharashtra (India)
14.	Katchal Micro Tall	KMT	Nicobar Islands (India)
15.	Perka Green Tall	PRGT	Nicobar Islands (India)
16.	Horned Cocos Tall	HCT	Andaman Islands (India)
17.	East African Tall	EAT	Kenya
18.	Fiji Tall	FJT	Fiji
19.	West African Tall	WAT	Côte d' Ivorie
20.	Surinam Tall	SUT	Surinam
<i>Dwarf accessions</i>			
21.	Kenthali Orange Dwarf	KTOD	Karnataka (India)
22.	Andaman Yellow Dwarf	AYD	Andaman Islands (India)
23.	Chowghat Green Dwarf	CGD	Kerala (India)
24.	Chowghat Orange Dwarf	COD	Kerala (India)
25.	Malayan Green Dwarf	MGD	Malaysia
26.	Niu Oma Yellow Dwarf	NOYD	Samoa
27.	Nikkore Orange Dwarf	NKOD	Papua New Guinea
28.	Hari Papua Orange Dwarf	HPOD	French Polynesia
29.	Niu Leka Green Dwarf	NLGD	Fiji

each of Kenthali Orange Dwarf and Andaman Yellow Dwarf and one of Chowghat Green Dwarf), for pooled DNA analysis. The pooled samples were used to identify putative markers for the plant type trait in coconut.

2.3. RAPD analysis

Initially, RAPD analysis was performed using 200 different decamer oligonucleotide primers (Series A–G, K, L and BA) obtained from Operon Technologies Inc. (Alameda, CA, USA) on the two (tall and dwarf) DNA pools. PCR reactions were conducted in volumes of 15 μ l containing 35 ng genomic DNA, 10 μ M primer, 10 mM of each dNTPs (M/s MBI Fermentas), 10 \times buffer (10 mM Tris–HCl, pH 8.3) and 5 Units of *Taq* DNA polymerase (M/s MBI Fermentas). The amplification conditions were: an initial denaturation step (94 °C for 5 min), followed by 39 cycles at 94 °C for 1 min, 42 °C for 1 min and 72 °C for 1 min 30 s, terminating with a final extension at 72 °C for 10 min. The amplified products were run on 1.2% agarose gel, stained with ethidium bromide and photographed on a digital gel documentation and image analysis system. Reproducible bands were scored visually. Each polymorphic primer was tested at least thrice to determine whether both the polymorphism and the banding patterns were reproducible. The polymorphic bands were then validated in individual tall and dwarf accessions.

2.4. Cloning of polymorphic fragments and sequencing

For the conversion of RAPD polymorphisms to SCAR marker, specific bands directly were excised from the agarose gel and purified using QIAquick gel extraction kit (Qiagen). The collected fragments were ligated into pTZ57R/T PCR cloning vector (Fermentas). The recombinant plasmids were transformed into *Escherichia coli* strain (DH5 α). Clones of polymorphic RAPD marker were then sequenced at the sequencing facility at SciGenom (Kochi, India).

2.5. Designing of SCAR primers and PCR analysis

Based on the sequence information of the cloned fragments, SCAR primer sets were designed using Primer3 (<http://frodo.wi.mit.edu/primer3>) and synthesized commercially (Sigma). The designed SCAR primers were employed to amplify the genomic DNAs of tall and dwarf coconut accessions. PCR reactions were conducted in volumes of 20 μ l containing 35 ng genomic DNA, 2 μ M of the primer, 10 mM of each dNTPs (M/s MBI Fermentas), 10 \times buffer (10 mM Tris–HCl, pH 8.3) and 5 Units of *Taq* DNA polymerase (M/s MBI Fermentas). The amplification conditions were: an initial denaturation step (94 °C for 5 min), followed by 35 cycles at 94 °C for 30 sec, 66 °C for 1 min and 72 °C for 1 min, terminating with a final extension at 72 °C for 10 min. The amplified products were run on 1.2% agarose gel, stained with ethidium bromide and photographed on a digital gel documentation and image analysis system.

2.6. Assessment of purity of hybrids using the SCAR marker

The SCAR marker was validated in the progenies of a D \times T cross (CGD \times WCT). Artificial pollination of CGD mother palms, with pollen of WCT, was carried out during January–March 2007 for the production of F₁ hybrid. Only palms showing typical characters of West Coast Tall and Chowghat Green Dwarf (Ratnambal et al., 1995) were selected for the crossing programme. The mature seed nuts were harvested during 2008, and sown in the nursery. One-year-old hybrid seedlings were planted in the field at a spacing of 7.0 meters during August 2009. DNA was extracted from parents and

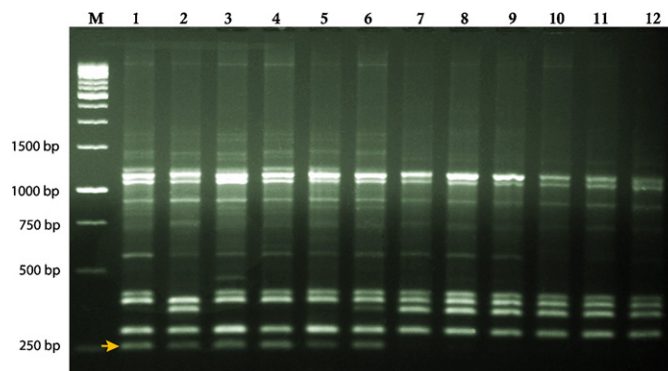


Fig. 1. RAPD banding pattern of pooled and individual tall and dwarf palms with the primer OPA9. Arrowhead indicates polymorphic band specific to tall accessions. M: 1 Kb ladder, Tall Samples: Lanes 1–Tall pool, 2–WCT, 3–FJT, 4–WAT, 5–SKGT, 6–SUT, Dwarf samples: Lanes 7–Dwarf pool, 8–KTOD, 9–AYD, 10–CGD, 11–COD, 12–MGD.

progenies. A total of seven progenies from the cross were evaluated for hybridity using the designed SCAR primer pair. Two selfed progenies were also included in this study.

3. Results

3.1. DNA extraction

High molecular weight genomic DNA was isolated from the spindle leaves of coconut accessions. DNA yield of 400–600 ng of DNA per 100 mg of tissue was obtained using the rapid extraction procedure. An absorbance ratio (A₂₆₀/A₂₈₀) of 1.6–1.8 indicated that there were no significant levels of proteins/polysaccharides contamination.

3.2. Screening RAPD markers linked to plant type trait

Among the 200 RAPD primers initially screened in the pooled DNA approach, one primer (OPA09) showed well reproducible polymorphic bands between the tall and dwarf plant type pool. These candidate marker identified by pooled DNA analysis were validated in individual tall and dwarf coconut palms representing different geographic regions (Table 1) to confirm their linkage to the plant-type trait. The marker OPA09₂₆₀ was present exclusively in tall accessions and absent in the dwarf cultivar (Fig. 1).

3.3. Conversion of the RAPD marker into SCAR marker

The putative marker amplified by the primer OPA 09 was excised, purified, cloned and sequenced. BLAST results revealed that the 260 bp sequence (Genbank accession no. JX406850) did not have similarity with any known nucleotide sequences. Based on the sequence of unique RAPD amplicon, a pair of SCAR oligonucleotide primers (Table 2), which could amplify ~260 bp of the genomic DNA of tall coconut accessions, was designed. Using the primer pair, PCR was performed in individual tall and dwarf coconut accessions. A single, distinct and brightly resolved band of ~260 bp was obtained only with tall accessions, while no amplification product was obtained with dwarf accessions (Fig. 2A and B). Thus, the RAPD marker, OPA09₂₆₀, was successfully converted into a SCAR marker.

3.4. Assessment of hybrids using the SCAR marker

A single, distinct and brightly resolved band of ~260 bp was obtained only with the tall parent (WCT) and the hybrids of the

Table 2
Details of tall-cultivar specific SCAR primer designed and utilized in the present study.

Name of the SCAR primer	Sequence of the SCAR primer (5'–3')	Length (bases)	T_m (°C)	Annealing temperature (°C)
COC1F	CCATCCCTCTGCCATCC	17	56	66
COC1R	GGTAGGTGCGGAAAAGG	17	54	

D × T cross. No band was noticed for the dwarf parent (CGD) or selfed progenies (Fig. 3).

4. Discussion

Presently, selection of hybrid seedlings in coconut nurseries relies on morphological markers, some of them being days taken for germination, vigour of seedlings in terms of leaf production, higher collar girth during a specific duration and petiole colour. The selection of hybrids by petiole colour (Bourdeix, 1988) has limitations as it is reliable only if progenitors homozygous for yellow, red or green petiole are used. Furthermore, many of the varieties and hybrids are phenotypically less distinct at early stages making morphological

selection and evaluation more difficult. Identification of genuine D × T (Dwarf × Tall) hybrids in the nursery stage is still more difficult task if the female parent is a green dwarf in which case, petiole colour is not useful for selection of hybrids. Though widely adopted and practiced, purity assessments based on morphology is often affected by environment, besides the higher requirement of time and resources. Identification of molecular markers for distinguishing plant-type character, therefore, is imperative for isolating true-to-type high-yielding tall and hybrid lines in the early stage of coconut breeding programmes which would be helpful in the commercial hybrid seedling production activities in coconut. Differentiation of dwarf, tall and hybrid types in the nursery based on combined application of morphological and molecular markers

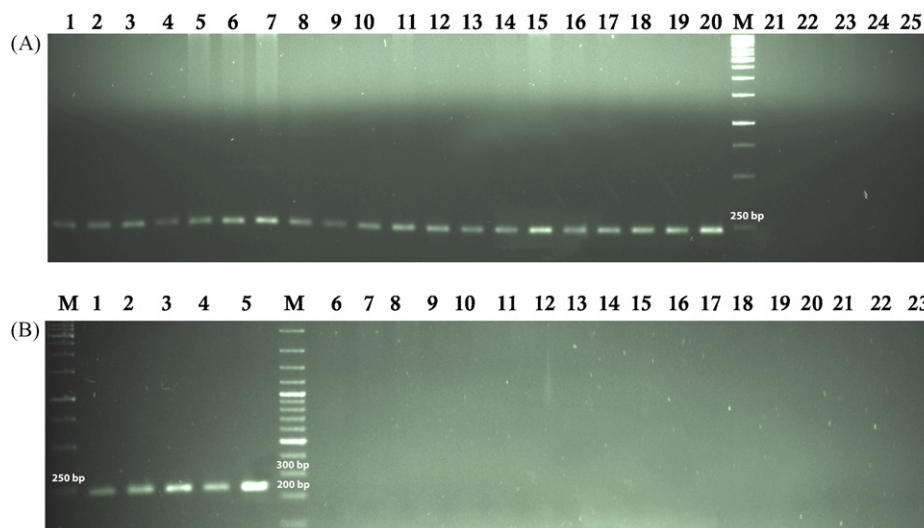


Fig. 2. (A and B) Validation of tall palm-specific SCAR marker. (A) Tall accessions: Lanes 1-FJT, 2-WAT, 3-SKGT, 4-WCT, 5-GPNT, 6-AYRT, 7-LMT, 8-LCT, 9-LCGT, 10-EAT, 11-SUT, 12-KPDT, 13-KMT, 14-PGT, 15-ANRT, 16-BDKT, 17-BENT, 18-TPPT, 19-KGT, 20-HCT; M: 1 Kb ladder; Dwarf accessions: 21-KTOD, 21-AYD, 23-CGD, 24-MGD, 25-COD, (B) M: 1 Kb ladder; Tall accessions: 1-FJT, 2-WAT, 3-SKGT, 4-WCT, 5-GPNT; M: 100 bp ladder; Dwarf accessions: 6-KTOD, 7-KTOD, 8-AYD, 9-AYD, 10-CGD, 11-CGD, 12-COD, 13-COD, 14-MGD, 15-MGD, 16-NOYD, 17-NOYD, 18-NKOD, 19-NKOD, 20-HPOD, 21-HPOD, 22-NLGD, 23-NLGD.

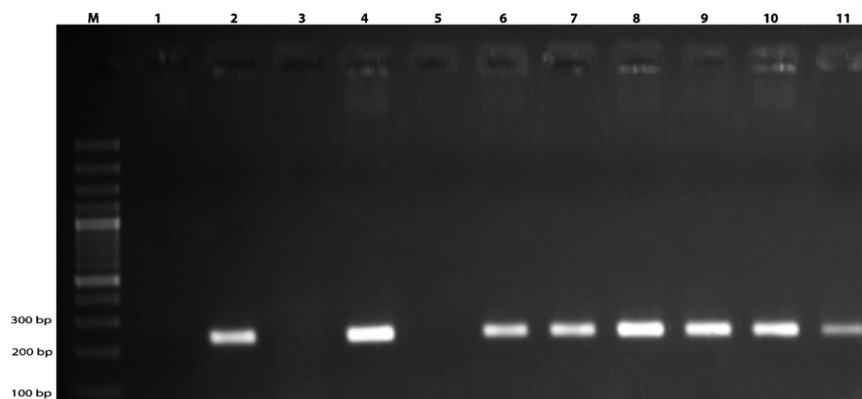


Fig. 3. PCR identification of D × T hybrids with SCAR marker. M: 100 bp ladder; lane 1: CGD (female parent); lane 2: WCT (male parent); lanes 4, 6, 7, 8, 9, 10, 11: CGD × WCT hybrid seedlings; lanes 3 and 5: CGD selfed progenies.

would be expected to ensure quality of planting material in terms of genetic purity.

In this study, we have described a technique of DNA pooling and RAPD technique to identify markers linked to tall-type palm trait in coconut. The methodology of pooling DNA for isolation of trait-specific markers has been reported earlier in Chinese jujube (Peng et al., 2001), Ponkan mandarin (JinPing et al., 2009) and betel vine (Khadke et al., 2012) and this methodology proved to be effective and practical in coconut too.

The fidelity of RAPD as a genetic marker technique has been questioned because of the numerous technical disadvantages associated with the RAPD technique. The reproducibility of RAPD is affected by many factors: the quality of template DNA, the concentration of primer and template, the different sources of DNA polymerase, etc. The reproducibility and reliability of RAPD assays can be significantly improved by the conversion of RAPD to SCAR markers, by developing longer, and hence, more specific primers from RAPD sequences. The linkage of the SCAR marker to tall-type palm trait in coconut was validated by screening the designed primer pair in a range of tall and dwarf coconut accessions of different geographical origins indicating the usefulness of the SCAR makers for marker-assisted breeding.

In earlier studies, RAPD markers have been utilized for genetic diversity studies of coconut accessions (Ashburner et al., 1997; Upadhyay et al., 2004), but there are no reports of tagging of plant-type trait in coconut using molecular marker technology as in other plant species such as *Brassica rapa* (Muangprom and Osborn, 2008), *Brassica napus* (Foisset et al., 1995), *Prunus* (Manago and Bliss, 1998), wheat (Sourdille et al., 1998) and banana (Damasco et al., 1996).

In conclusion, a marker related to tall-type plant trait has been identified using RAPD markers. The SCAR primers designed using this sequence variation was found to be specific for tall accessions making the technique more stringent and specific when compared with the RAPD marker. Studies of inheritance of plant-type character in coconut has occupied a major place in coconut breeding programmes as most of these programmes aim in developing a $D \times T$ or $T \times D$ hybrids. The SCAR marker can be utilized to distinguish selfed progenies in $D \times T$ crosses, as presented in this study. Also, during selection of pure dwarf progenies in nurseries, the SCAR marker would be of immense use in culling out of out-crossed seedling progenies resultant of tall pollen contamination. Thus, the SCAR marker for distinguishing tall and dwarf coconut accessions would give headway in establishing further varietal identity, achieving varietal purity within germplasm and ensuring the quality of planting material in hybrid planting material production in coconut.

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