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ARTICLE

Application of RAPD markers in hybrid verification in coconut

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Abstract – *Coconut palms are classified into two major types, viz., 'talls' and 'dwarfs', which mainly differ in their pollination behavior of cross- and self-pollination, respectively. Due to this difference, getting true-to-type progenies of desirable tall and dwarf cultivars has always been a challenge. The conventional practice of selection of seedlings based solely on morphological traits often results in selection of out-crossed seedlings and undesirable off-types. In the present investigation, RAPD markers for the tall/dwarf trait were identified in coconut using a bulked DNA approach. Screening of tall and dwarf palm bulk DNA with 200 primers revealed a RAPD primer OPBA3 which was able to clearly differentiate both the tall and dwarf bulks. For validation, the primer was used to screen individual tall and dwarf coconut palms representing different geographic regions. The primer was also used to screen the parents and validate hybrids of Dwarf x Tall crosses.*

Key words: *Arecaceae, hybridity, genetic purity, molecular markers.*

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is considered as "the symbol of the tropics" and "the tree of heaven" in view of its position as a key plantation crop of the tropics and also its versatile contribution to humankind. Almost every part of the coconut tree is used either to generate income or to meet the food requirements of rural communities (Persley 1992). Coconut products provide food, shelter and energy to farm households, and can be made into various commercial and industrial products. When strategically used, it can increase food production, improve nutrition, create employment opportunities, enhance equity and help to preserve the environment. In addition to being a food crop, coconut is considered as an industrial crop. The coconut industry is intimately connected with the economic and domestic life of the inhabitants of coconut-growing states. The coconut palm has been known to exist in most regions of the tropics from pre-historic times. It is now considered that the coconut palm might have originated independently in the Pacific and also the Indo-Atlantic oceanic basins (Gunn et al. 2011). Humans might have brought about its spread to other regions of the world through ships, and nature through sea currents.

One of the key determinants of success in breeding programs for development of new cultivars of any plant species is the reproductive or mating system of the species. Coconut cultivars are generally classified into tall and dwarf types based on the stature and size of the palm. Tall palms are referred to as var. *typica* and dwarf palms referred to as var. *nana*. Tall coconuts are fast growing and are mainly allogamous (cross-fertilizing) and constitute the polymorphic populations (Rao et al. 2005). Since they are hardy and thrive in a wide range of environmental conditions, they are the most commonly cultivated palms in all the coconut-growing areas of the world. Dwarf palms are mainly autogamous (self-fertilizing) and are characterized by their short stature. They are quicker to come to bearing (3-4 years) and easier to harvest, but are short lived. They have a thin trunk without a swollen base or bole, with closely arranged leaf scars and they yield heavily. Dwarfs are identified mainly by the colour of their nuts. They are presumed to have originated from tall palms, either through mutation or through inbreeding in talls (Nambiar and Swaminathan 1961).

Hybrids are mostly inter-varietal crosses between the two morphological forms of coconut. They exhibit earliness in flowering, increased nut yield, and higher copra production and they give better quality copra and oil as compared to the parents. In coconut, hybrids are mainly produced in two ways, with tall as female parent and dwarf as male parent (T x D) or dwarf as female parent and tall as male parent

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(D x T). In addition, inter-varietal hybrids like Tall x Tall (T x T) and Dwarf x Dwarf (D x D) are also produced. The manifestation of hybrid, vigour or heterosis in coconut was first reported in 1932 in inter-varietal crosses involving the tall variety as the female and dwarf variety as male in which the seedlings exhibited hybrid vigour. Hybrid coconut cultivation offers an opportunity to farmers to increase coconut yields.

Quality planting material is the key to success in the cultivation of any crop and its contribution to better performance is exponentially higher especially in the case of perennial crops like coconut, where yield can be realized only after a long period. Currently, morphological descriptors are used for varietal identification, description and seed purity and hybrid assessment in coconut (Arunachalam and Rajesh 2008). Hybrid seedlings produced are selected in the nursery by either of two markers, *viz.,* germination speed and petiole colour. Selection of hybrids by petiole colour, which is the most widely used marker to select hybrid seedlings in the nursery stage, is reliable only if progenitors homozygous for yellow, red or green petiole are used. Many of the varieties and hybrids are phenotypically less distinct at the seedling stage, making morphological evaluation more difficult. It is not possible to identify true hybrids if both the progenitors are of same petiole colour, thus making it very difficult to distinguish true hybrids from self-pollinated progenies. Though widely adopted and practiced, purity assessments based on morphology are often not reliable. Morphological traits are subjected to environmental effects, further limiting their use. This requires development of an alternative technique that can offer an efficient and reliable assessment of genetic purity.

Molecular markers offer an attractive option to morphological markers in coconut hybrid production programmes. Simple sequence repeats (SSR), also known as microsatellites, are widely accepted as reliable markers. SSR-marker-based approaches have been used to develop identification methods for coconut varieties and hybrids of T X D crosses [involving Sri Lanka Tall, Sri Lanka Green Dwarf and Sri Lanka Yellow Dwarf] (Perera 2010) and D x T crosses [involving Chowghat Green Dwarf and West Coast Tall] (Rajesh et al. 2012). Also, in a recent study, we have developed a RAPD-derived SCAR marker for the tall-type palm trait in coconut (Rajesh et al. 2013). However, these markers are cultivar specific and specific markers need to be developed for specific crosses.

As coconut is a long duration perennial crop, it is imperative to undertake strict quality control. The aims of the present study were to develop a set of molecular markers which could be universally used for differentiation of tall and dwarf coconut cultivars and utilize the selected markers in hybrid seedling purity assessments.

MATERIAL AND METHODS

Plant materials

The plant material used for molecular marker analysis consisted of tall and dwarf accessions representing different geographic regions (Table 1). Also, parents and hybrid of three D x T crosses, *viz.,* Chowghat Green Dwarf (CGD) X West Coast Tall (WCT), Chowghat Orange Dwarf (COD) x Klapawangi Tall (KWGT) and Malayan Yellow Dwarf (MYD) x San Ramon Tall (SNRT), were utilized.

DNA isolation and pooling

High quality DNA was isolated from spindle leaves of coconut accessions using a rapid method (Rajesh et al. 2013). The isolated DNA was air-dried and dissolved in 0.75 ml TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA).

Initially, we constituted two genotypic DNA pools: a tall plant type pool including five tall cultivars (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 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 tall and dwarf cultivars in coconut.

RAPD analysis

We performed RAPD analysis on the two (tall and dwarf) DNA pools using 200 different decamer oligonucleotide primers (Series A, B, C, D, E, F, G, K, L and BA; Operon Technologies Inc., Alameda, CA, USA). PCR reactions were conducted in volumes of 15 µl containing 35 ng genomic DNA, 10 µM primer, 10 mM of each dNTP (M/s MBI Fermentas), 10X 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 1min, 42 °C for 1 min and 72 °C for 1 min 30 sec, terminating with a final extension at 72 °C for 10 min. The amplified products were run on 1.2 percent agarose gel, stained with ethidium bromide and photographed on a digital gel documentation and image analysis system. Reproducible bands were scored visually. We tested each polymorphic primer at least three times to ensure reproducibility of polymorphism and the banding patterns. We then carried out validation of the polymorphic bands in individual tall and dwarf accessions.

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

Sl. No.	Name of the accessions	Abbreviation	Origin
Tall accessions			
1.	West Coast Tall	WCT	Kerala (India)
2.	Sakhigopal Tall	SKGT	Odisha (India)
3.	Gangapani Tall	GPNT	Andhra Pradesh (India)
4.	Ayiramkachi Tall	AYRT	Tamil Nadu (India)
5.	Laccadive Ordinary Tall	LCT	Lakshadweep Islands (India)
6.	Laccadive Micro Tall	LMT	Lakshadweep Islands (India)
7.	Fiji Tall	FJT	Fiji
8.	West African Tall	WAT	Côte d' Ivorie
9.	Philippines Ordinary Tall	PHOT	Philippines
10.	San Ramon Tall	SNRT	Philippines
11.	Klapawangi Tall	KWGT	Malaysia
Dwarf accessions			
1.	Kenthali Orange Dwarf	KTOD	Karnataka (India)
2.	Andaman Yellow Dwarf	AYD	Andaman Islands (India)
3.	Chowghat Green Dwarf	CGD	Kerala (India)
4.	Chowghat Orange Dwarf	COD	Kerala (India)
5.	Malayan Orange Dwarf	MGD	Malaysia
6.	Malayan Yellow Dwarf	MYD	Malaysia
7.	Sri Lankan Green Dwarf	SLGD	Sri Lanka

Assessment of hybrid purity using RAPD markers

Once validated, these markers were utilized for screening the parents and hybrids to confirm their utility as a hybrid authentication tool. The parents and hybrid of three D x T crosses. *viz.,* Chowghat Green Dwarf (CGD) X West Coast Tall (WCT), Chowghat Orange Dwarf (COD) x Klapawangi Tall (KWGT) and Malayan Yellow Dwarf (MYD) x San Ramon Tall (SNRT), were utilized for assessment of hybrid purity.

RESULTS AND DISCUSSION

In spite of its simplicity and rapidity, there are a few technical limitations associated with the RAPD technique, especially reproducibility. The use of short decamers as primers and the resulting low annealing temperatures have raised questions as to its fidelity as a molecular marker. Therefore, for the present study, we have raised the annealing temperature for RAPD analysis to 42 ^{o}C. Also, we have chosen highly reproducible and specific amplicons from 500 bp to 1500 bp for analysis.

RAPD analysis used 200 primers to allow identification of markers, which exhibited amplification of bands unique to tall and dwarf coconut accessions. Among the RAPD primers initially screened in the pooled DNA approach, one primer (OPBA03) showed well reproducible polymorphic bands between the tall and dwarf plant type pool (Figure 1). These candidate markers identified by pooled

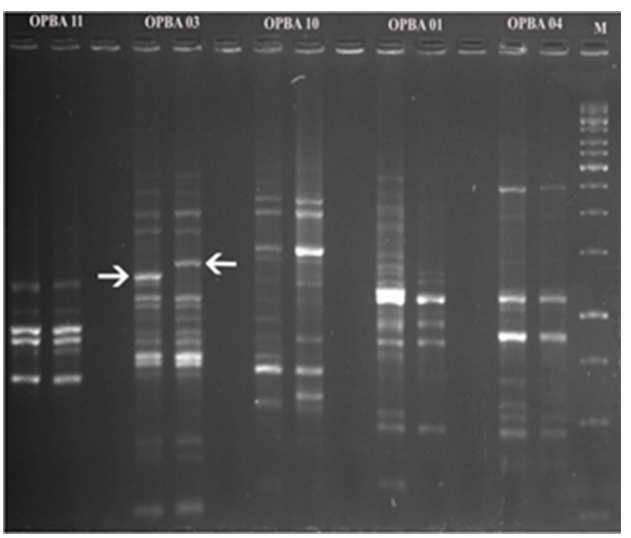

Figure 1. RAPD banding pattern of tall and dwarf bulks with different primers of OPBA series. Arrowheads indicate polymorphic bands of OPBA03 distinguishing tall and dwarf pooled DNA.

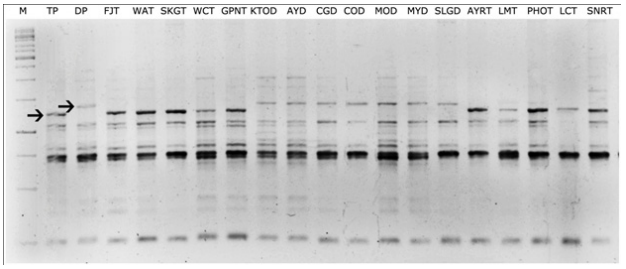

Figure 2. RAPD banding pattern on individual tall and dwarf palms with the primer OPBA3. Arrowheads indicate polymorphic bands distinguishing tall and dwarf DNA. M: 1 Kb ladder, TP: Tall pooled sample, DP: Dwarf pooled sample.

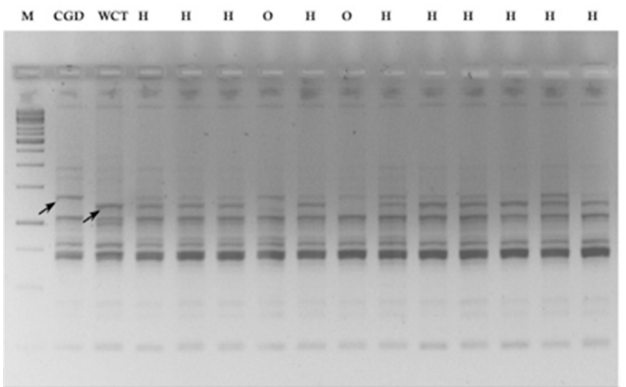

Figure 3. RAPD banding pattern of CGD x WCT parental palms and their hybrids with the primer OPBA3. Arrowheads indicate polymorphic bands distinguishing tall and dwarf parents used in hybrid production. M: 1 Kb ladder, H: Hybrids, O: Off-types

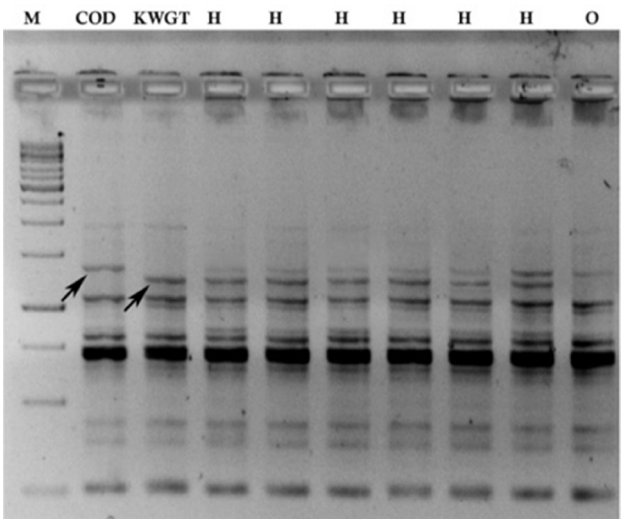

Figure 4. RAPD banding pattern of COD x KWGT parental palms and their hybrids with the primer OPBA3. Arrowheads indicate polymorphic bands distinguishing tall and dwarf parents used in hybrid production. M: 1 Kb ladder, H: Hybrids, O: Off-types.

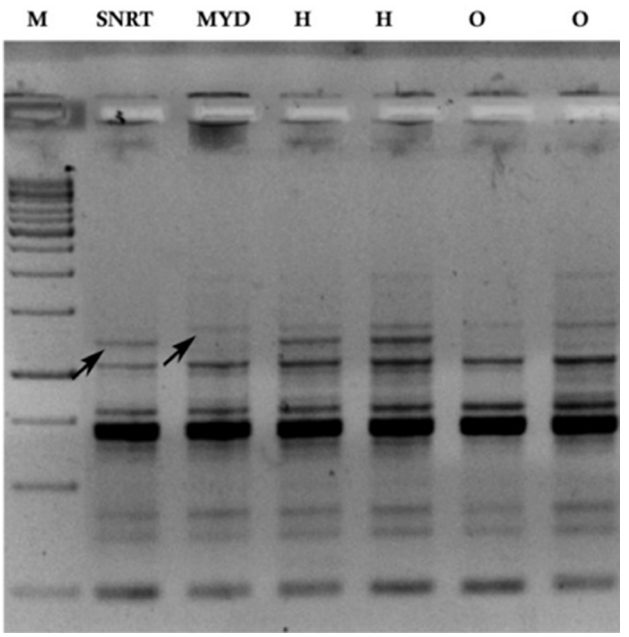

Figure 5. RAPD banding pattern of MYD x SNRT parental palms and their hybrids with the primer OPBA3. Arrowheads indicate polymorphic bands distinguishing tall and dwarf parents used in hybrid production. M: 1 Kb ladder, H: Hybrids, O: Off-types.

DNA analysis were validated in individual tall and dwarf coconut palms from different parts of the world (Table 1) to confirm their linkage to the tall/dwarf trait. The primer OPBA3 produced a band of around 1200 bp present only in tall palms and a band of around 1300 bp present only in dwarf palms (Figure 2).

Assessment and maintenance of genetic purity of hybrids play a crucial role in successful adoption of hybrid coconut technology. The polymorphisms observed between the tall and dwarf coconut cultivars were used as markers for hybrid identification in three crosses, *viz.,* CGD x WCT, COD x KWGT and MYD x SNRT (Figure 3, 4, 5). Comparing the RAPD banding pattern of parents with respective hybrids, genuine hybrids were confirmed since the hybrids possessed the unique bands of both the parental palms. Off-types (selfed progenies of the mother palm) could also be detected using these markers.

Presently, breeders select hybrid seedlings in coconut nurseries relying solely on morphological markers, e.g., days taken for germination, vigour of seedlings in terms of leaf production, higher collar girth over a specific duration and petiole colour. Though widely adopted and practiced, purity assessments based on morphology are often affected by environment, in addition to time and resources. Moreover, seedlings selected based on these traits might not be true hybrids, which may adversely affect breeding programmes in coconut, a long duration, perennial crop. Therefore,

identification of molecular markers for distinguishing plant-type trait is imperative for isolating true-to-type high-yielding hybrid lines in the early stage of coconut breeding programmes which would be helpful in commercial hybrid seedling production in coconut. Molecular markers are known to be reliable for hybridity testing of economically important crops. The differentiation of dwarf, tall and hybrid types in the hybrid nursery based on combined application of morphological and molecular markers would ensure the maintenance of quality planting material.

In this study, we have described a DNA pooling and RAPD technique to identify markers linked to the tall-type palm trait in coconut. The methodology of pooling DNA for isolation of trait-specific markers has already been reported in other plants like Chinese jujube (Peng et al. 2001), Ponkan mandarin (Jin Ping et al. 2009) and betel vine (Khadke et al. 2012). The RAPD technique is one of the most widely used and its popularity derives from its simplicity and rapidity, the requirement for only a small quantity of DNA, and the ability to generate numerous polymorphisms. RAPD markers have been utilized for hybrid authentication in Chrysanthemum (Huang et al. 2000), capsicum (Ilbi 2003) and cotton (Ali et al. 2008, Asif et al. 2009). The issues of the reproducibility and reliability of RAPD assays can be significantly improved by the conversion of RAPD into SCAR markers, by developing longer and, consequently, more specific primers from RAPD sequences. The leads obtained in this way would pave the way for the development and validation of SCAR markers linked to the plant-type trait in coconut and its utilization in marker-assisted breeding.

In conclusion, in the present study, RAPD markers related to the tall and dwarf trait were identified and these markers were utilized to identify D x T hybrids, which can ensure effective selection by plant breeders. Use of these markers would allow identification of true hybrids in the nursery stage and identification and removal of selfed progenies from coconut seedling nurseries, thus substantially saving time and resources.

Aplicação de marcadores RAPD na verificação híbrida em côco

Resumo - *Coqueiros são classificados em dois principais tipos: 'gigantes' and 'anões', os quais diferem principalmente em seus comportamentos de polinização; cruzamento e auto-polinização, respectivamente. Devido a essa diferença, obter progênies verdadeiras de cultivares gigantes e anões desej*áveis tem sido um desafio*. A prática da seleção convencional de* seedlings *baseada somente em caracteres morfológicos resulta, frequentemente, em seleção de* seedlings *de tipos indesejáveis. No presente trabalho, marcadores RAPD para o caráter gigante/anão foram identificados em coqueiros usando abordagem de* bulk *de DNA*. Screening *no* bulk *de DNA de plantas gigantes e anãs com 200* primers *revelaram um* primer *RAPD OPBA3 capaz de diferenciar claramente ambos os bulks. Para sua valida*ção*, esse* primer *foi usado para triagem de coqueiros gigantes e anões de diferentes regiões geográficas. Esse mesmo* primer *foi usado também na triagem de parentais e para validar híbridos dos cruzamentos de gigante x anão.*

Key words: *Arecaceae, híbrido, pureza genética, marcadores moleculares.*

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