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Hybrid Testing and Variety Identification of Coconut (*Cocos nucifera* L.) in Sri Lanka Using Microsatellite Markers

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Abstract

Currently, there is no reliable method for confirmation of the identity of coconut cultivars and the legitimacy of coconut hybrids. This makes serious problems in coconut breeding and seed production as the identity of coconut cultivars/hybrids can only be approximated at the very late stages of the growth based on reproductive traits owing to long juvenile period and the perennial nature of coconut. A microsatellite (SSR) marker based approach was used to develop an identification method for coconut varieties; Sri Lanka Tall, Sri Lanka Green Dwarf and Sri Lanka Yellow Dwarf, the key parents in the breeding programmes and tall x dwarf hybrids among them, using 18 coconut specific SSR primers. The validity of using yellow colour petiole as a phenotypic marker for the identification of dwarf yellow was also investigated. Two SSR primers, namely CAC20 and CNZ6 have exhibited the potential for distinguishing coconut varieties used as parents in the breeding programme and for confirming hybridity. It was revealed that yellow colour petiole is not a reliable marker for identification of dwarf yellow variety.

Keywords: coconut, *Cocos nucifera,* genetic diversity, hybrid testing, plant identification, SSRs-microsatellites.

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Introduction

The coconut palm, *Cocos nucifera* L. is a major plantation crop and one of the most important palms of the wet tropics. Coconut is the most extensively grown and used nut in the world, playing a significant role in the economical, cultural and social life of over 80 coconut growing countries. The demand for coconut is mainly for oil, copra, desiccated coconut and fresh nuts for consumption though coconut has a variety of other uses (Harries, 1995).

In Sri Lanka, there are three commercially available recommended coconut cultivars, viz. Sri Lanka Tall x Sri Lanka Tall (SLT x SLT), Sri Lanka Green Dwarf x Sri Lanka Tall (SLGD x SLT) and Sri Lanka Yellow Dwarf x Sri Lanka Tall (SLYD x SLT). These three cultivars are mainly produced at an Isolated Coconut Seed Garden (ISG) (Liyanage *et al.*, 1988), where highly selected SLT, SLGD and SLYD palms were planted. The SLT is predominantly cross-pollinating and SLGD and SLYD are predominantly self-pollinating. Thus, SLGD and SLYD matured inflorescences are forced opened and are manually emasculated to avoid self-pollination. This process ensures SLGD x SLT and SLYD x SLT hybrid seeds from the dwarf palms and SLT x SLT seeds from SLT palms by natural cross pollination within the ISG. The fidelity of the crosses at ISG is highly important, but 100% legitimacy of hybrids could not be obtained as a result of sporadic, accidental natural opening of dwarf inflorescences resulting in contaminant pure dwarf seeds. Further, accidental mixing of seed lots when harvesting and transporting and mislabelling of seedbeds during nursery stage are also problems. In addition, misidentification of varieties/cultivars in long term breeding trials pose serious problem in coconut breeding.

Yellow colour petiole is generally considered a phenotypic maker for SLYD. Therefore, yellow petiole producing seedlings are considered a contaminant in coconut nurseries and are discarded. However, the accuracy of considering yellow colour as a marker for SLYD has not been proved by genetic analysis. Further, there is no such phenotypic marker for SLGD. Germination speed being comparatively faster in dwarfs than hybrids, it may be used as a criterion to identify contaminating dwarfs, yet due to large variation of germination speed among individuals of dwarfs, use of this parameter for variety identification has again limited use. Therefore, currently there is no reliable approach to confirm the identity of commercially available coconut cultivars in Sri Lanka.

Establishment of a reliable method for identification of coconut varieties with greater accuracy is extremely necessary. DNA fingerprinting provides the basis for such an approach. Out of many molecular marker methods, microsatellites (SSRs) (Powell *et al.*, 1996) is considered the identical tool for variety identification and hybrid testing particularly because they are co-dominant and multi-allelic. The objective of this study was to develop a SSR based DNA finger printing approach for identification of coconut varieties, cultivars and hybrids. The validity of using yellow colour petiole as a phenotypic marker for identification of SLYD is also investigated.

Materials and methods

SLT, SLGD, SLYD, SLGD x SLT, SLYD x SLT, SLT x SLT and seedlings having yellow colour petioles germinated in SLYD x SLT hybrid seedbeds were used for the study. Total genomic DNA was isolated from fresh coconut leaves of 20 individuals each from each cultivar, variety and hybrid and from 50 yellow colour petiole produced seedlings, using standard CTAB DNA extraction protocol (Perera *et al.*, 1999). Initial primer screening was carried out based on the allelic patterns produced for different varieties represented by three individuals per variety, using 10 primers developed at Scottish Crop Research Institute, Scotland (Perera *et al.*,1999, 2003) and 5 SSR primers developed at Long Ashton Research Station, UK, (Rivera *et al.*, 1999). PCR reactions were carried out as described in Perera *et al.*, (1999), but without radio labelling. The selected primers were then tested on 20 samples each of all varieties, cultivars and hybrids to

confirm the uniformity of results. PCR products were separated on 6% PAGE in 1 x TBE buffer and stained with Silver nitrate.

Results

Of the primers tested, two primer pairs namely CAC20 (Perera *et al.*, 2003) and CNZ6 (Rivera *et al.*, 1999; Teulat *et al.*, 2000) produced unique markers for different varieties. Primer CAC20 produced two specific markers (alleles) for SLT and their sizes were estimated as 132bp and 134bp. They were present either as single allele as homozygote or as double allele as heterozygote. In contrast, CAC20 produced one unique allele each for SLGD (136bp) and for SLYD (138bp) (Figure 1). The hybrids between SLGD x SLT and SLYD x SLT analysed were readily distinguishable in the gel by the presence of either of SLT specific allele (132bp or 134bp) with either the SLGD specific allele (136bp) or with the SLYD specific allele (138bp) in the heterozygote state.

The primer CNZ6 distinguished SLT by a single allele designated "a" and SLGD by a single allele designated "b" (size of the markers are to be decided). However, the primer CNZ6 generated four alleles for SLYD, designated "b", "c" ,"d" and "e", the alleles "c", "d" and "e" being exclusive to SLYD. These alleles were present in SLYD both in homozygote state and heterozygote state (Figure 2). As allele "a" was exclusive to SLT, hybrids were recognizable by the presence of "a" allele with any other alleles. The SLGD x SLT and SLYD x SLT hybrids were indistinguishable by CNZ6 primer.

It was revealed that some yellow colour petiole producing seedlings analyzed in this study using CNZ6, were hybrids between SLYD x SLT, as they had both SLT allele and one of the SLYD specific allele in heterozygote state (Figure 3). The same result was confirmed by the CAC20 primer. The percentage of hybrid seedlings among yellow coloured seedlings was 11%.

Discussion

The results indicate that the primer CAC20 can be successfully used to uniquely distinguish SLT, SLGD and SLYD varieties and resulting hybrids, SLGD x SLT and SLYD x SLT. This approach had been successfully used for identification of soybean varieties (Rongwen *et al.*, 1995), Navy bean varieties (Graham *et al.*, 1994), apple varieties (Tancred *et al.*, 1994) and rose varieties (Torres *et al.*, 1993). The presence of either 132bp or 134bp alleles in homozygote or heterozygote state confirms the SLT and SLT x SLT, when presence of 136bp and 138bp alleles in their homozygote state confirms the varieties SLGD and SLYD respectively. The presence of either 132bp or 134bp with 136bp allele in heterozygote state confirms the SLGD x SLT hybrid. Similarly presence of either 132bp or 134bp allele with 138bp allele in heterozygote state confirms the SLYD x SLT hybrid. Therefore this primer can be used to confirm legitimate hybrids and to uniquely identify parental varieties used in the breeding programme and the resulting hybrids. This approach can also be effectively used to confirm the identity of materials during accidental mixing of seed lots or mislabelling of seedbeds, and during seed and seedling certification. In addition, monitoring of crossing programmes for breeding trials and identification of contaminants in the segregating population for genetic mapping trials can accurately be done using this method to minimize errors which could usually detects only long years after planting. The primer, CNZ6 can also be used to separate dwarf varieties from SLT and confirm the hybrids, though this primer can not distinguish between SLDG x SLT and SLYD x SLT. However, both these primer can be used to eliminate all or most of the problems described in the introduction with greater accuracy, by testing only a single or few samples from a seed bed or seedling lot in doubt.

Although, yellow colour petiole is generally considered a phenotypic marker for SLYD and is used to identify contaminant dwarfs from hybrid seed beds, this study revealed that not all yellow seedlings are pure SLYDs. In the Philippines also, green colour has been used as a phenotypic marker to detect non-hybrids from the Tagnanan tall x Malayan red dwarf, but later DNA fingerprinting of this

Figure 1. Allelic distribution pattern among SLT, SLDG, SLDY, SLDG x SLT and SLDY x SLT with CNZ6

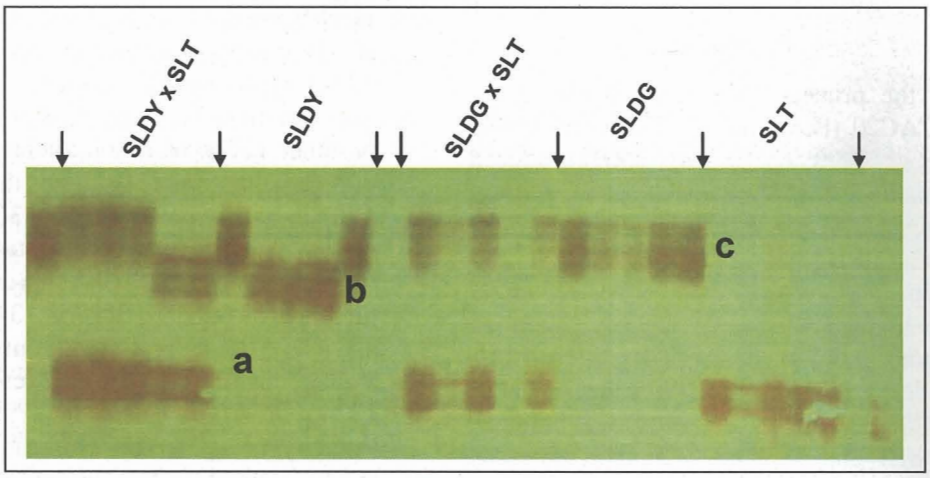


Figure 2. Allelic distribution pattern among SLT, SLDG, SLDY, SLDG x SLT and SLDY x SLT with CAC20

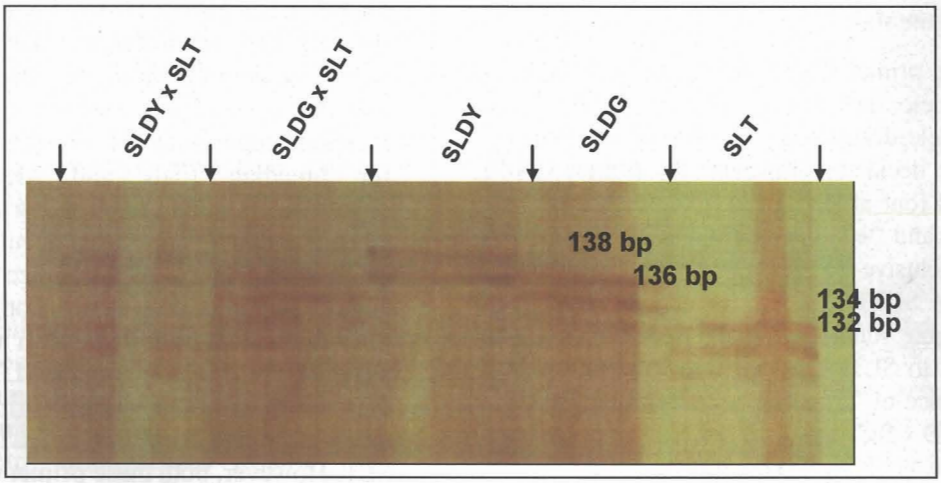
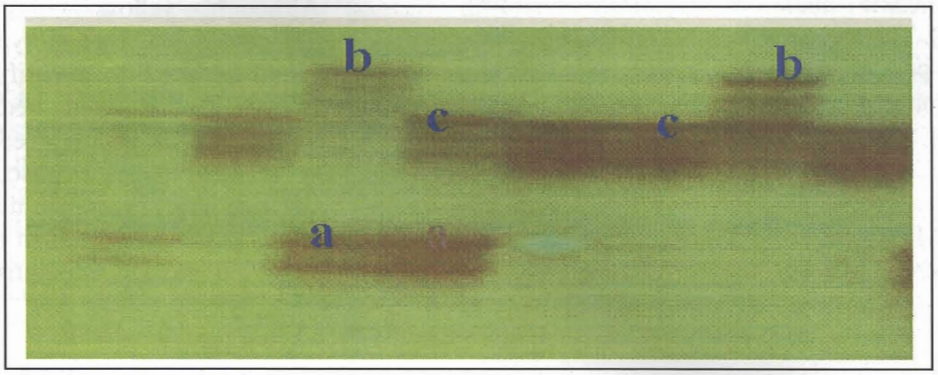


Figure 3. CNZ 6 primer showing two hybrid seedlings among yellow colour seedlings appeared in hybrid seedbeds (a specific to SLT, b & C specific to SLDY)



varieties revealed that not all green seedlings are illegitimates. Similarly out of fifty yellow seedlings tested with CAC20 and CNZ6 primers, 14 yellow colour seedlings were identified as hybrid seedlings. Thus, the DNA marker approach developed in this study can be used to recover about 11% of valuable hybrid seedlings which were usually discarded as contaminant. However, as the cost of a seedlings does not compensate the cost of a diagnostic PCR reaction at current rates, this would have some practical application in future. The yellow colour petiole producing seedlings appeared hybrids using SSR diagnosis were planted in the field along with rest of the other yellow petiole producing seedlings in order to confirm the results obtained by DNA finger printing.

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