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SHORT RESEARCH ARTICLE

Molecular marker assisted confirmation of hybrids in coconut (*Cocos nucifera* L.)

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Abstract

Hybrid authentication is important in the selection of good quality hybrid seedlings in coconut before distribution to various stakeholders. Fourteen coconut specific SSR primer pairs were initially used to screen parental lines used in coconut hybrid nut production for polymorphism. Primers capable of differentiating parents, CncirE2 and CnCirE10 were then utilized to screen seedlings in the coconut nursery to identify true hybrids based on the complementary banding pattern of both male and female parents

Keywords: Authentication, coconut, hybridity, SSR markers

Coconut (*Cocos nucifera* L.) is an important multipurpose tree of the humid tropics that supports millions of people in more than 93 countries for their livelihood. It has been traditionally used for its copra for oil and varied products from endosperm, tender nut water, fibre, husk, and leaves. Quality planting material is crucial for successful cultivation of any crop especially in the case of perennial crops like coconut where yield can be realized only after a long period. Coconut exhibits considerable genetic variations and can be propagated only through seed, therefore, the selection and use of planting material of true hybrids assume considerable importance. The palm contributes to yield for many years and the full bearing capacity becomes known only 10 to 15 years after planting and therefore, a superior quality palm is to be ensured for higher economic yield for growers. Since coconut cultivation involves extensive pre-bearing investment, more importance must be given to the selection and use of the right type of planting material. Among the different discriminators used to identify the coconut cultivars, time taken to sprouting is the most useful and important discriminator. Leaf production rate, leaf length and width and time taken to first leaf splitting are important indicators when used in combination. Hybrid coconut seedlings are identified in the nursery stage based on morphological characters like early germination, petiole colour and seedling vigour. Hybrid seedlings of Dwarf x Tall, can be selected based on the exclusive characteristics of the male parent *viz*., colour of petiole, length and breadth of leaves and leaflets, etc. One year old coconut seedlings usually exhibit hybrid vigour for collar girth, number of leaves, length and breadth of leaves and leaflets, etc. One of the most commonly used distinguishing characteristic is petiole colour and hybrids are usually selected based on the colour of petiole and collar girth.

Accidental mixing of seed lots while harvesting and transporting and mislabelling of seedbeds during nursery stage are major problems. Further, misidentification of cultivars in long term breeding trials create serious problem in coconut breeding programme. At present, there is no reliable method for confirmation of the identity of coconut cultivars and the legitimacy of coconut hybrids. This is a major problem 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.

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DNA markers are ideal for characterization and identification of genotypes for rapid and early verification of true hybrids at the seedling stage. Simple sequence repeats (SSRs) or microsatellites are the most effective and suitable DNA markers not only for hybridity testing but also for genomic research hence, they are commonly utilized in testing the heterozygosity or homozygosity of the hybrids in different crops (Cordiero et al. 2000; Patella et al. 2019; Dimitrijevic and Horn 2018; Johnson et al. 2019; Bharat et al. 2021). The objective of this study was to develop SSR-based DNA finger printing approach for identification of coconut hybrids.

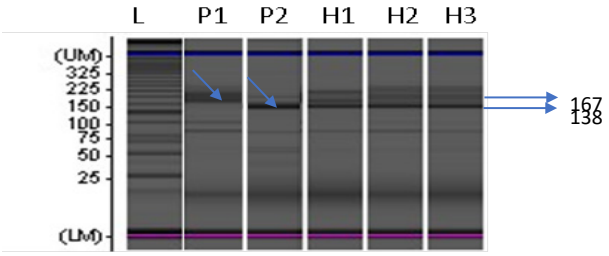
The plant materials used for hybrid authentication using molecular markers consisted of tall and dwarf parents and their offspring collected from ICAR-CPCRI, Kasaragod, Kerala, India. A total of 20 parental lines and 45 progenies were used for the study (Table 1).

DNA was extracted from spindle leaves of coconut parental lines and hybrid seedlings using a rapid method (Rajesh et al. 2013). The extracted DNA was airdried and dissolved with 0.75 ml TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA purity was checked by running the agarose gel (0.8 per cent) stained with ethidium bromide and visualized in a gel documentation system.

Initially, all the parental palms used in hybrid seed production were screened with a set of 14 coconut specific SSR markers, for distinguishing COD, WCT and LCT parental lines used for hybrid coconut seedling production (Rajesh 1 et al. 2008). PCR reactions were performed in volumes of 20 µL and contained genomic DNA (35 ng), 10 mM of each dNTPs (MBI Fermentas), 0.2 µM primer (Sigma), 3 Units of Taq DNA polymerase (MBI Fermentas) and 10X buffer [10 mM

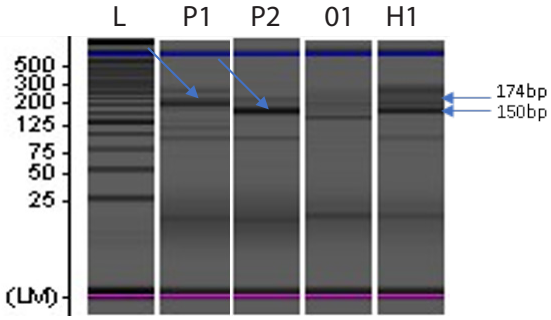
Table 1. List of hybrids and their parental lines used in the study

S. No.	Hybrid	Female parent	Male parent
Kera Sankara (WCT x COD)			
1.	1,2,3	WCT550	COD J 73
2.	4,5,6	WCT579	COD J 179
3.	7,8,9,10	WCT 532	COD J 172
4.	11,12,13	WCT 555	COD J 68
5.	14,15,16	WCT 568	COD J 68
6.	17,18,19	WCT 597	COD J 151
7.	20,21,22	WCT 482	COD J 57
8.	23,24, 25	WCT 550	COD J 68
Chandra Sankara (COD x WCT)			
9.	26,27,28	COD J 65	WCT 525
10.	29,30,31	COD J 81	WCT 525
11.	32,33,34	COD J 73	WCT 525
Chandra Laksha (LCT x COD)			
12.	35,36,37,38,39	LCT 42	COD J 62
13.	41,42,43,44,45	LCT 3	COD J 163



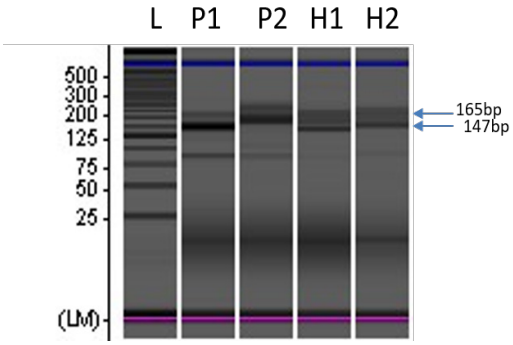
L- 25 bp ladder; P1 - WCT parent (♀); P2 - COD parent (♂) ; H1 to H3: Hybrids

Fig. 1. SSR marker CnCirE2 profile confirming hybridity of coconut hybrids (WCT X COD)



L- 25 bp ladder; P1 -LCT parent (♀); P2 - COD parent (♂); O1 - off-type; H1: Hybrid

Fig. 2. SSR marker CnCirE2 profile confirming hybridity of coconut hybrids (LCT X COD)



L- 25 bp ladder; P1 - COD parent (♀); P2 - WCT parent (♂); H1 and H2: Hybrids

Fig. 3. SSR marker CnCirE2 profile confirming hybridity of coconut hybrids (COD X WCT)

Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$. The amplification conditions followed were: initial denaturation step at 94 °C for 2 minutes, 39 cycl es at 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute 30 seconds and concluding with a final extension at 72 °C for 10 minutes. The amplified products were analyzed using an automated microchip based electrophoresis system MultiNA (Shimadzu biotech, Japan). The highest peak spotted by the fragment analyzer was scored for the presence of the expected band for each primer pair. Primers which are polymorphic and capable of differentiating the parental palms were then utilized for

hybrid purity assessment studies.

Screening of hybrids using molecular markers

The validated markers were utilized for screening the hybrids derived from particular parental combinations to confirm their utility as a hybrid authentication tool. The parents and hybrid of three crosses viz., Chowghat Orange Dwarf (COD) X West Coast Tall (WCT), West Coast Tall (WCT) X Chowghat Orange Dwarf (COD) and Laccadive Ordinary Tall (LCT) X Chowghat Orange Dwarf (COD) were utilized for assessment of hybrid purity.

The current study utilized the SSR marker technique for the identification of coconut hybrids along with its parental lines. Microsatellite analysis was carried out as described in Rajesh 2 et al. (2008). The primers showing polymorphism between the parental lines were selected and utilized to survey their F1 hybrids for parentage confirmation. Out of the 14 microsatellite markers tested, two markers (CnCirE2 and CnCirE10) could unambiguously differentiate the tall and dwarf parents (Supplementary Table S1). These selected markers, which exhibited amplification of allele's specific to a parental line, were then used to screen the 45 hybrids of three crosses in order to test the reliability of the SSR markers.

Based on the complementary banding patterns between the hybrids and their parents, the SSR marker 'CnCirE2 and CnCirE10' were identified as the two specific markers to distinguish the F1 hybrids. The CnCirE2 amplified a specific allele of size 167bp in F1 hybrid, female parent (WCT) and not its pollen parent (COD). Allele size of 138bp was observed in F1 hybrid and pollen parent (COD) (Fig. 1.A). Further, the CnCirE2 also amplified the allele of size 165bp in pollen parent (WCT) and 147bp in female parent (COD) in another cross Kera Sankara (WCT x COD). The allele size of 165 and 147bp was amplified in the F_1 hybrid (Fig. 1B). Similarly, an allele size of 174bp was amplified in female parent (LCT) and 150 bp was amplified in pollen parent (COD). F1 hybrid of these crosses amplified both 150bp and 174bp (Fig. 1C). Hence, the presence of both female and male parent alleles was noted as a result of crossing between two parents (F_1 hybrid).

Microsatellites being co-dominant, a true hybrid would possess the banding pattern of both the parents. The two microsatellites selected conclusively displayed the complementary banding patterns of both the parents (Fig. 1), confirming the role of microsatellites as a promising approach in purity monitoring of coconut hybrids. This technique proved that SSR markers can be successfully applied to distinguish and identify the hybrids from their parental lines. Since, SSR markers is co-dominant and had more polymorphism than most other DNA markers, it promoted the application of microsatellites in fingerprinting (Ashikawa et al. 1999) of crop varieties. Genetic purity testing by SSR markers has also been demonstrated in maize (Wang et al. 2002) and in rice (Nandakumar et al. 2004). Rajesh et al. (2012) used SSR markers for identification of D x T hybrids ((CGD x WCT) Kalpa Sankara) in coconut. RAPD markers were utilized earlier in our laboratory to identify D x T hybrids in coconut. Preethi et al. (2016) used EST-SSR markers in determining the genetic purity of different hybrids in coconut.

Off-types or selfed progenies could also be detected using these markers. Currently, breeders select hybrid seedlings in coconut nurseries relying exclusively 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. Coconut seedlings selected based on these morphological 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 isolating true to type high yielding hybrid lines in the early stage of coconut breeding programmes is important which would be helpful in commercial hybrid seedling production in coconut. The identification and differentiation of tall, dwarf and hybrid types in the coconut nursery based on combined application of morphological and molecular markers would ensure the maintenance of quality planting material.

Supplementary material

Supplementary Table S1 is provided.

Authors' contribution

Conceptualization of Research (RS, KS, MKR); Designing of the experiments (RS, KS, MKR); Contribution of experimental material (KS, VN); Execution of lab experiment and data collection (RS, MKR); Analysis of data and interpretation (RS, MKR, KS); Preparation of the manuscript (RS, MKR, KS, VN).

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Supplementary Table S1. Details of SSR primers used and primers showing polymorphism between male and female parents

S No.	Primers	Sequence (5′–3′)		Annealing Temperature(°C)	Polymorphism between male and female parents
		Forward	Riverse		
1	CnCir A3	AATCTAAATCTACGAAAGCA	AATAATGTGAAAAAGCAAAG	52	-
2	CnCir A9	AATGTTTGTGTCTTTGTGCGTGTGT	TCCTTATTTTTCTTCCCCTTCCTCA	59	-
3	CnCir B6	GAGTGTGTGAGCCAGCAT	ATTGTTCACAGTCCTTCCA	58	-
4	CnCir B12	GCTCTTCAGTCTTTCTCAA	CTGTATGCCAATTTTTCTA	56	-
5	CnCir C3	AGAAAGCTGAGAGGGAGATT	GTGGGGCATGAAAAGTAAC	58	-
6	CnCir C7	ATAGCATATGGTTTTCCT	TGCTCCAGCGTTCATCTA	58	-
7	CnCir C12	ATACCACAGGCTAACAT	AACCAGAGACATTTGAA	54	-
8	CnCir E2	TCGCTGATGAATGCTTGCT	GGGGCTGAGGGATAAACC	55	+
9	CnCir E10	TGGGTTCCATTTCTTCTCTCATC	GCTCTTTAGGGTTCGCTTTCTTAG	57	+
10	CnCir E12	TCACGCAAAAGATAAAACC	ATGGAGATGGAAAGAAAGG	58	-
11	CnCir F2	GGTCTCCTCTCCCTCCTTATCTA AC	CGACGACCCAAAACTGAAC	58	-
12	CnCir G11	AATATCTCCAAAAATCATCGAAAG	TCATCCCACACCCTCCTCT	58	-
13	CnCir H4	TTAGATCTCCTCCCAAAG	ATCGAAAGAACAGTCACG	54	-
14	CnCir H7	GAGATGGCATAACACCTA	TGCTGAAGCAAAAGAGTA	58	-