Research Article

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Identification and utilization of informative EST-SSR markers for genetic purity testing of coconut hybrids

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Abstract

Coconut palms are categorized into two forms, *viz*., 'talls' and 'dwarfs' which are being utilized to produce hybrids through the process of inter-varietal or intra-varietal crosses. Hybrid coconut seedlings are generally identified and selected based on morphological traits by plant breeders, which is quite difficult and requires expertise. Even minor errors in identification may adversely affect breeding programs in coconut, which is spread over many decades. In this study, we have utilized thirty EST-SSR markers, derived from existing coconut leaf transcriptome data, for screening polymorphism between eighteen coconut parental lines. The polymorphic primers capable of differentiating the parental palms were then utilized successfully for assessment of purity of hybrids derived from these parents. Thus, the current study demonstrates the utility of EST-SSR markers in determining the genetic purity of hybrids in coconut.

Keywords: Coconut, hybrids, genetic purity, EST-SSR

Introduction

Generating and testing hybrid varieties of coconut are currently a major field of research in many countries with the objectives of increasing yield of nuts, oil content and also tolerance to abiotic and biotic stresses. There are many hybrids being developed and researched upon to cater to the climate, soil conditions and needs of each individual location. The two major varieties of coconut palms are 'talls' and 'dwarfs' (Narayana and John, 1949) with dwarfs (even though fewer than 5 per cent of the world coconut population) being in higher demand for genetic studies due to their quick emission of inflorescence and early germination (Bourdeix *et al.*, 2008). Talls take a longer time to flower (~6 years) but live much longer (~100 years) when compared to dwarfs (~60 years). Talls (var. *typica*) and dwarfs (var. *nana*) also differ in their breeding behaviour with the talls being allogamous (cross-fertilizing) and dwarfs being autogamous (self-fertilizing) (Arunachalam and Rajesh, 2008). Inter-varietal crosses between a dwarf male parent with a tall female parent (T x D) as well as tall male parent with a dwarf female parent (D x T) and intra-varietal crosses (T x T and D x D) are methodologies followed for the development of hybrids (Arunachalam and Rajesh, 2008).

Hybrid varieties that provide better resistance to various diseases and enhanced yield have been successfully developed in coconut. Kalpa Sankara, a hybrid resistant to root (wilt) disease has been derived by crossing Chowghat Green Dwarf (CGD) and West Coast Tall (WCT) (Nair *et al.*, 1996). Hybrids developed between Vanuatu Tall (VTT) and Rennell Island Tall (RIT) have been reported to possess better resistance towards coconut foliar decay disease, which is endemic to Vanuatu in the South Pacific (Labouisse *et al.*, 2011). Recently, Kalpa Samrudhi, a cross between Malayan Yellow Dwarf (MYD) and WCT, has been developed which

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provides a much higher nut yield, copra content as well as oil output when compared to its parents (Jerard *et al.*, 2015).

Even though the development of hybrids has contributed significantly for the increased productivity of coconut, the timely production and ample supply of hybrid seedlings, which are genetically pure, to the farmers is the key factor determining the success of hybrid technology in this crop. Morphological descriptors currently used for seed purity assessment in coconut include petiole colour, days taken for germination, seedling vigour and higher collar girth (Rajesh *et al.*, 2014). Although hybrid purity assessments based on morphology are extensively taken up, they are often affected by environment; in addition, requirement for time and resources for such an assessment is tremendous. Selection by petiole colour, which is generally utilized marker to select hybrid seedlings in nurseries, is authentic only if parents used are homozygous for red, yellow or green petiole (Rajesh *et al.*, 2014). Some of the drawbacks of utilizing morphological traits for genetic purity testing of coconut hybrids are that they are cumbersome and subjected to environmental influences. Furthermore, many of the varieties and hybrids are phenotypically less distinct resulting in difficulty in accurate morphological evaluation.

DNA-based markers, because of their rapidity in estimation, ease of use and cost-effectiveness, have become indispensable for use in variety identification, diversity and linkage-mapping studies. Among the common molecular markers, SSR (simple sequence repeat) are generally preferred due to their abundance, co-dominant inheritance, presence over the whole genome, higher reproducibility, multi-allelic nature, hyper-polymorphism and high transferability across species/genera (Varshney *et al.*, 2005). SSRs have been developed and utilized in coconut for genetic diversity studies (Rivera *et al.*, 1999; Perera *et al.*, 2000; Meerow *et al.*, 2003; Rajesh *et al.*, 2008 a,b).

The use of SSRs for the authentication/ differentiation of hybrids is a widely accepted procedure in many crops (Antonova *et al.*, 2006; Sundaram *et al.*, 2008; Naresh *et al.*, 2009) and has been previously used in coconut too. SSR-based identification of Kalpa Sankara hybrids has been reported by Rajesh *et al.* (2012). In a cross between Sri Lanka Yellow Dwarf (SLYD) and Sri Lanka Tall (SLT), progenies with yellow colour were removed as selfed progenies based on visual observations (since SLYD petioles are yellow in colour), but SSR analysis later on proved that at least 11 per cent of the discarded yellow seedlings were actually hybrids (Perera, 2010).

Although genomic SSR markers have been utilized for genetic purity studies in plants traditionally, their high cost and time involved in this process have restricted their utilization. The number of SSR markers available in coconut is limited. With the exponential accumulation of data in EST databases, EST-derived SSRs (EST-SSRs) are being utilized these days for various molecular studies. EST-SSRs are also advantageous in that these SSRs might be from gene sequences that are functional, ESTs being located in the coding region of a gene. EST-SSR markers have been utilized earlier in genetic purity assessment of annual crops like safflower (Naresh *et al.*, 2009) and castor (Pranavi *et al.*, 2011; Gouri Shankar *et al.*, 2013), but there are no such reports in perennial tree crops. In this study, we aim to identify novel markers that could decisively validate different coconut hybrids through the use of EST-SSRs.

Materials and methods

Plant materials

The plant materials used for hybrid authentication using molecular markers consisted of tall and dwarf parents and their offsprings collected from the ICAR-CPCRI Farm, Kasaragod, Kerala, India. A total of 18 parental lines and 103 progenies were used for the study (Table 1).

DNA isolation

DNA was extracted from spindle leaves of parental palms and their progenies following the modified method of Rajesh *et al.* (2013). To check the DNA purity, it was run in 0.8 per cent agarose gel, stained with ethidium bromide and visualized in a gel documentation system.

Assessment of parental polymorphism using EST-SSR markers

Initially, all the parental palms used in hybrid seed production were screened using the 30 novel

Table 1. Details of parental palms used for hybrid authentication studies and EST-SSR primers showing parental polymorphism

Cross no.	Parents		EST-SSR primer showing polymorphism
1	CGD	Chowghat Green Dwarf	CnKGDEST126
	WCT	West Coast Tall	CnKGDEST117
2	MYD	Malayan Yellow Dwarf	CnKGDEST126
	TPT	Tiptur Tall	
3	COD	Chowghat Orange Dwarf	CnKGDEST126
	WCT	West Coast Tall	
4	GBGD	Gangabondam Green Dwarf	CnKGDEST130
	PHOT	Philippines Ordinary Tall	
5	GBGD	Ganga Bondam Green Dwarf	CnKGDEST130
	LCT	Laccadive Ordinary Tall	
6	LCT	Laccadive Ordinary Tall	CnKGDEST130
	CCNT	Cochin China Tall	
7	GBGD	Gangabondam Green Dwarf	CnKGDEST130
	FJT	Fiji Tall	
8	WCT	West Coast Tall	CnKGDEST126,
	COD	Chowghat Orange Dwarf	CnKGDEST117
9	LCT	Laccadive Ordinary Tall	CnKGDEST117
	COD	Chowghat Orange Dwarf	
10	COD	Chowghat Orange Dwarf	CnKGDEST117
	CCNT	Cochin China Tall	
11	CGD	Chowghat Green Dwarf	CnKGDEST117
	CCNT	Cochin China Tall	
12	MYD	Malayan Yellow Dwarf	CnKGDEST117
	SNRT	San Ramon Tall	
13	MOD	Malayan Orange Dwarf	CnKGDEST117
	SNRT	San Ramon Tall	
14	MGD	Malayan Green Dwarf	CnKGDEST117
	CCNT	Cochin China Tall	
15	CRD	Cameroon Red Dwarf	CnKGDEST117
	CCNT	Cochin China Tall	
16	COD	Chowghat Orange Dwarf	CnKGDEST117
	SNRT	San Ramon Tall	
17	GBGD	Gangabondam Green Dwarf	CnKGDEST117
	SNRT	San Ramon Tall	
18	MYD	Malayan Yellow Dwarf	CnKGDEST117
	CCNT	Cochin China Tall	

EST-SSR primers (Table 2), which were mined from leaf transcriptome data of Chowghat Green Dwarf cultivar (Rajesh *et al*., 2015) as per the procedure reported in Preethi *et al.* (2014). 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 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 cycles 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 amplicons were separated on 3 per cent agarose gel and photographed on a digital gel documentation and image analysis system after staining with ethidium bromide. Polymorphic primers capable of differentiating the parental palms were then utilized for hybrid purity assessment studies.

Results and discussion

Thirty novel EST-SSR primers were used to screen polymorphism among eighteen parental lines. Those primers capable of detecting polymorphism among the parental palms in a particular cross were selected (Table 1). Confirmation of the results was achieved through repeated testing. For all these markers, the alleles present in the parents were of different sizes and both parental alleles were detected in the hybrids, EST-SSRs being co-dominant markers.

The hybridity of 14 F_1 plants derived from CGD x WCT were tested through the use of CnKGDEST126 and CnKGDEST117 primers, which displayed polymorphism between the parental lines. Out of 14 F_1 progenies, a total of 11 were confirmed to be true hybrids while three were deduced to be selfed or off types using CnKGDEST117 primers (Fig. A). Out of a total of six F1 progenies tested from a cross between MYD and TPT, two offsprings were deduced to be offtypes and the other four as true hybrids using the primer CnKGDEST126 (Fig. 1B). In a cross between COD x WCT, two pure hybrids and two selfed F1 progenies were detected using the primer CnKGDEST126 (Fig. 1C). F_1 progenies of the

Table 2. List of EST-SSR primers used for the study and their details

Sl. No.	Primer Name	NCBI accession number	Repeat type	Forward primer (5′-3′)	Reverse primer (5′-3′)	Functional annotation	Annealing temperature (°C)
1	CnKGDEST81	KU999089	$(GCGACCT)_6$	TCGCCCAAAGCCACCCTCCTAT	TCGCCCGCAGGGAAAAATCCAC	DNA polymerase I	58
2	CnKGDEST82	KU999090	$(TC)_{21}$	AAAGAGTAGCGAAAGCAAGTTTCAAGC	TGGACAAAGAGACAGACAGACAGAC	Keratin-associated protein 10-12-like	60
3	CnKGDEST84	KX580069	$(AAT)_{13}$	TGAAGACGCGGGTGAGGTTGGA	GGGAGCCAAAGGTGTCAAGGCA	Leucine-rich repeat receptor-like protein kinase	59
4	CnKGDEST87	KX580070	$(TC)_{20}$	ACGGCTTCCTCTCAGCCTCCAA	TGCCACTTGCCGGTGAAAAAGGT	Glycosyl transferase	59
5	CnKGDEST96	KU999091	$(TTC)_{13}$	TGGGATAGACCTTGGTCTGTTGCTAT	TTCTCGCATCGTTCGTGATCCTG	Serine threonine protein	57
6	CnKGDEST100	KU999092	$(GAGGCG)_7$	TGGCCCTCAGCGAAAGGGAGAA	ACCGACGAGAATGGCGGTCTCT	50s ribosomal protein l3	59
7	CnKGDEST101	KX580071	$(TC)_{13}$	AGGCCTGGCACCACCTCTTCTT	ATCGAGGCAGCCCCACCTTACT	bhlh transcription factor-like protein	59
8	CnKGDEST103	KU999093	$(TA)_{23}$	ACCCCAATGCCCGTGTGTGAAC	AGGGCTAAGATTGCAGTGACCCT	Midasin	59
9	CnKGDEST95	KU999094	$(TC)_{20}$	ACGGCACCAATTGGGTCAGACG	TTGGTGCAGTTTCTTGGCCCCC	Phosphomethyl pyrimidine synthase, chloroplastic	58
10	CnKGDEST106	KU999095	$(AT)_{21}$	TCTGATGGCACCCGCATTGGAG	TCATCCAAGACTGCCACACGCC	ACT domain containing protein	59
11	CnKGDEST85	KU999096	$(CT)_{21}$	TGGATATCACAGCCCTTCCATGCT	GTTGCAGTTTGGTGCATGTGAAAGAT	Squalene synthase	57
12	CnKGDEST90	KU999097	$(TA)_{20}$	GGCACAACCAGTGTCTCTTTGGCA	GTGGCTTTGCCTCCCCATGCTT	F-box protein SKP2A-like	59
13	CnKGDEST98	KU999098	$(GT)_{23}$	AGACCCTCATGCACTAGGCCAC	TGGCCAACACACCAGGAATTTGT	Isovaleryl-CoA dehydrogenase, mitochondrial	58
14	CnKGDEST199	KU999099	$(GA)_{20}$	ACTTGTTGGGATAGGGTGCGGC	TCCACCATGCCCACAACAGTGC	Glutamate receptor 3.5 isoform X1	59
15	CnKGDEST107	KU999100	$(TC)_{21}$	TGTCCATGCTTTTGTGGATGTGGGT	TGTGTGTGTGTGTGTGTGTGTGT	Serine/arginine-rich splicing factor SR30	59
16	CnKGDEST119	KU999101	$(GA)_{21}$	CGCATGGGAGGCTGAGGCAAAA	AAGGGGCCTCTTCCCATGCCTT	Ethylene-responsive transcription factor ERF113	59
17	CnKGDEST123	KX580072	$(TC)_{20}$	GCAGTTTGACTGCTGCACTTTGCC	ACACACACACACACACACACACA	Early flowering 3-like isoform X1	59
18	CnKGDEST126	KU999102	$(TG)_{21}$	ACTCTGCTGCTTTTCCAGACAGGT	AGTTAACAGAATCACATTGGCGGACA	UV-damaged dna-binding	58
19	CnKGDEST136	KU999103	$(AG)_{22}$	AGCTCCAGTCCACCCACGGAAT	TCCTTCCCTCCATCATTCCCTCTCT	Abscisic acid 8-hydroxylase	58
20	CnKGDEST139	KU999104	$(TC)_{23}$	TCCACGGTGCCCCTATTGGTCA	ACGAGAAAGGAGACAAAATGGGGAAAA	Riboflavin biosynthesis protein riba	59
21	CnKGDEST142	KU999105	$(GGCGGA)_6$	TGCATAAACCGGGCAGTCGGTG	CCCCCGCCCATTGAAATCGGAA	Methyltransferase- like protein	59
22	CnKGDEST143	KU999106	$(TC)_{20}$	TCTTCTTCCTCGTCTCCTCCACCC	AGAGCTCTCCAGTGGCGACCAA	COP1-interacting protein-related	58
23	CnKGDEST91	KU999107	$(AAAAACA)_5$	TGCAGCCACATGCGTTCACAGA	AGCTGGGATGGAAAGCAAAGGGC	Peroxisome biogenesis protein 22-like	59
24	CnKGDEST115	KU999108	$(TGGAGC)_7$	AGGTTGGTTGAGGCCGGAGCTT	AGGTGCAACGGGAGCCTCATCT	Ubiquitin receptor RAD23b-like	60
25	CnKGDEST117	KX580073	$(TAGA)_{10}$	CCGCCTCGGTTCAACAAACCCA	TCCCCACCCCACAACCAAACCA	Inactive β-amylase 9	59
26	CnKGDEST137	KX580074	$(CATA)_{10}CC(CA)_{21}$	GCCTTGGTAAGCGTTGGAATGTCCT	AACCACATGGCCCCTACTCCCA	Sym-1 like protein	59
27	CnKGDEST122	KX580075	$(TC)_{25}$	TATGCTGGTGCGGAGAGTGGGA	CGCGTAATTGGCAACTGACATGGG	2-Hydroxyacyl-CoA lyase-like	59
28	CnKGDEST124	KX580076	$(AG)_{21}$	ACACACACACACACACACACA	TGGTTGTCCTTGGATTGCACATGGT	ADP-ribosylation factor GTPase-activating protein AGD13	59
29	CnKGDEST129	KX580077	$(AG)_{22}$	AGCTGCAGCACCGAGGATGAGA	TGCGTTTCATGCACTCACAAAAGG	Transcription factor MYB3- like	59
30							
	CnKGDEST130	KX580078	$(CT)_{27}$	GCCATTGGAGGCATGGAAGCCA	TCTCCTGCTGCCCTCTTCCCTT	GRF1-interacting factor like	59

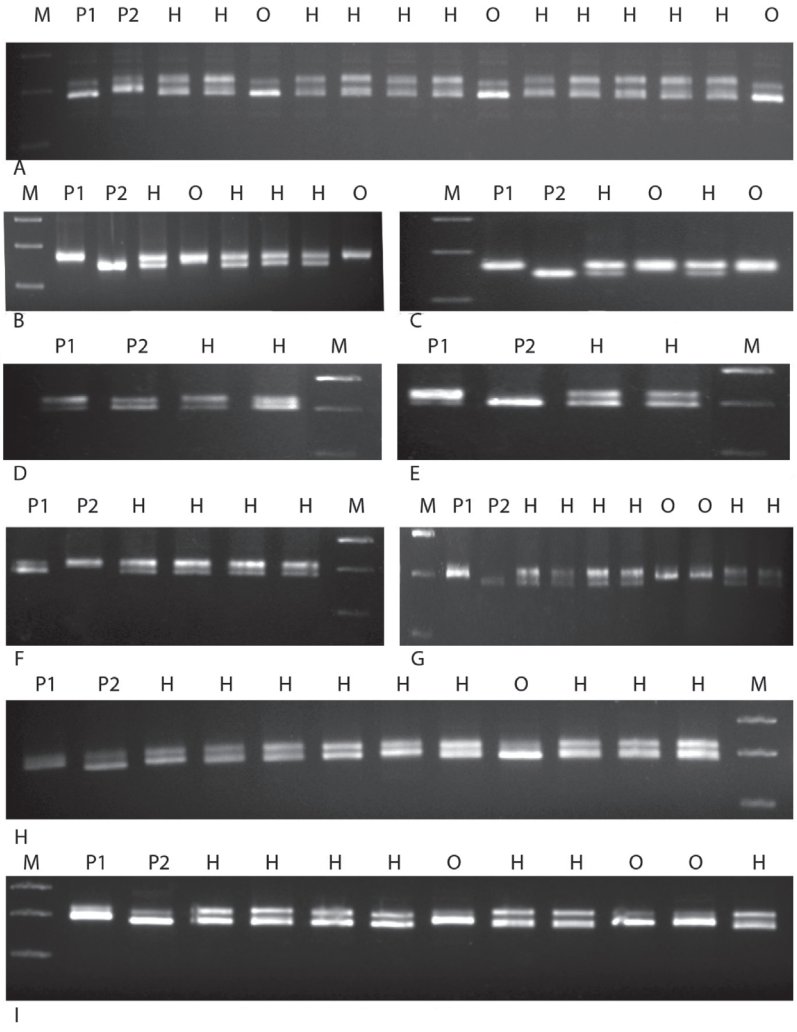

Fig. 1. Gel profile of coconut parents and their hybrids (M: 100bp ladder, P1: Female parent, P2: Male parent, H: Hybrids, O: Offtypes, A: CGD x WCT, B: MYD x TPT, C: COD x WCT, D: GBGD x PHOT, E: GBGD x LCOT, F: LCOT x CCNT, G: GBGD x FJT, H: WCT x COD, I: LCOT x COD)

crosses GBGD x PHOT (Fig. 1D), GBGD x LCOT (Fig. 1E) and LCOT x CCNT (Fig. 1F) were all confirmed to be true hybrids when checked with primer CnKGDEST130. Two selfed F_1 progenies were detected out of a total of eight probable hybrids in GBGD x FJT cross using the primer CnKGDEST130 (Fig. 1G). The primer CnKGDEST117 could aid in identifying one offtype from among ten F_1 progenies with the others confirmed as true hybrids in WCT x COD (Fig. 1H). LCT x COD cross revealed three offtypes and seven pure hybrids using the primer CnKGDEST117 (Fig. 1I).

Progenies of crosses between COD x CCNT (Fig. 2A), CRD x CCNT (Fig. 2B) and MYD x CCNT (Fig. 2C) showed true hybrids in all the lanes of the F_1 progenies used for testing with the primer CnKGDEST117. In CGD x CCNT (Fig. 2D) and MYD x SNRT (Fig. 2E), out of four progenies, two pure hybrids and two offtypes were identified using the primer CnKGDEST117. The same primer, CnKGDEST117, was used for the assessment of hybrid purity in MOD x SNRT (Fig. 2F) and MGD x CCNT (Fig. 2G) which showed that out of four F1 progenies, only one was a true hybrid with the others being offtypes. Assessment of progenies of COD x SNRT with the primer CnKGDEST117 revealed that there was an offtype among the four F_1 progenies (Fig. 2H). In the cross between GBGD

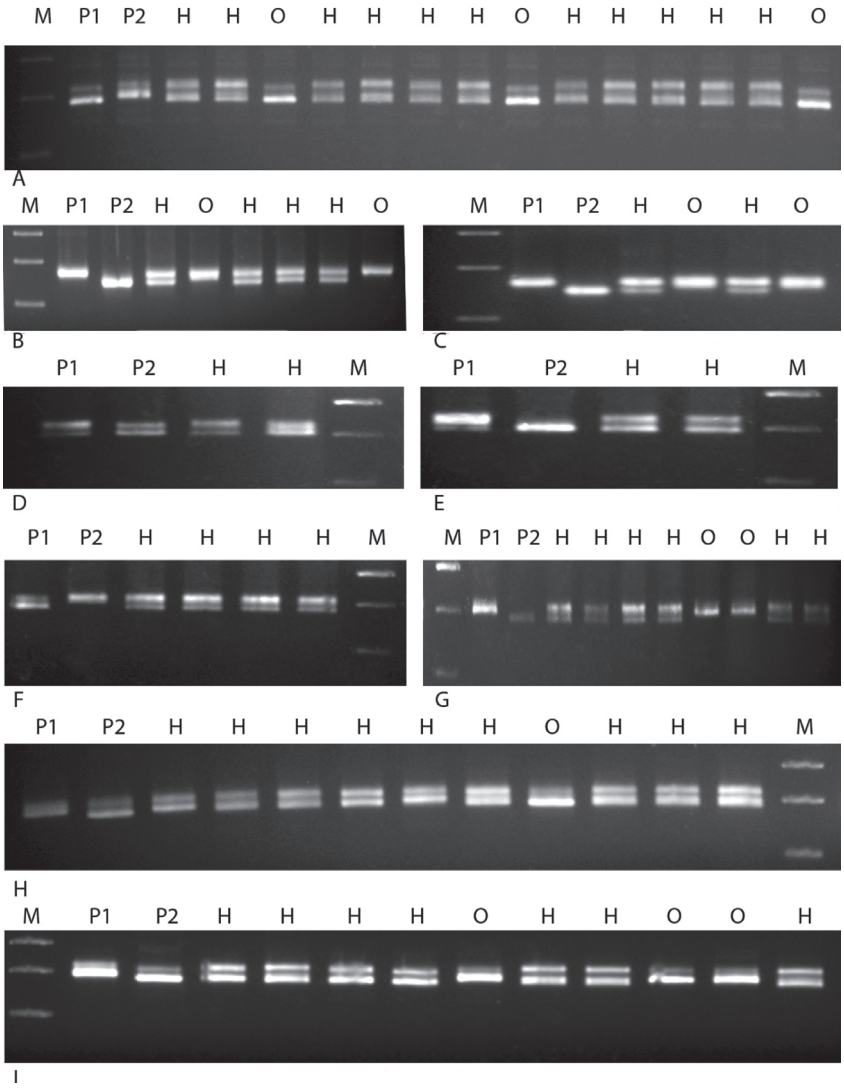

Fig. 2. Gel profile of parents and hybrids
(M: 100bp ladder, P1: Female parent, P2: Male parent, H: Hybrids, O: Offtypes, A: COD x CCNT, B: CRD x CCNT, C: MYD x CCNT, D: CGD x CCNT, E: MYD x SNRT, F: MOD x SNRT, G: MGD x CCNT, H: COD x SNRT, I: GBGD x SNRT)

and SNRT, when tested with the primer CnKGDEST117, a total of three selfed progenies were detected among the four F_1 progenies tested (Fig. 2I).

Identifying hybrids in an early stage is of prime importance for breeders; using morphological markers for this purpose is an unreliable method to identify a hybrid mainly due to the fact that the morphological traits are limited, display dominant expression thus reducing statistical capability, are influenced by the environment and they might change according to the development phase of the plant (Kumar *et al.*, 2009). Despite these disadvantages, morphological traits like petiole colour, days taken for germination, seedling vigour in terms of leaf production and higher collar girth over a specific duration are still utilized for identification of hybrids in coconut (Rajesh *et al.*, 2014). With reference to a perennial crop like coconut, it is also of utmost importance that proper hybrid identification be done at an early stage due to the long time that it takes to grow, flower and

bear fruit. Commercial hybrids are hugely popular in coconut with both public and private sectors being actively invovled in the development of hybrids. This necessitates strict quality control with respect to monitoring seed genetic purity at various production stages for the success of hybrid technology among stakeholders.

Presently, EST-SSRs have emerged as an important category of molecular markers due to their ease of availability, their hyper variability nature, their aptness for high throughput analysis, their high rate of polymorphism and cross-transferability in comparison to other available markers (Poczai *et al.*, 2013). EST-derived SSR markers possess great potential for use in marker assisted selection (MAS), for developing high yielding varieties, molecular mapping and quantitative trait loci (QTL) analysis (Varshney *et al.*, 2005). In coconut, they are few reports on identification of EST-SSR markers in coconut (Xiao *et al.*, 2013; Xia *et al.*, 2014). However, the present study is the first report of hybrid authentication studies in coconut utilizing EST-SSR markers. Furthermore, the markers identified through this study could be utilized in assessments of purity of hybrid seedlings and identification and subsequent elimination of selfed progenies from seedling nurseries, resulting in considerable economy with respect to time and resources.

Acknowledgements

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