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RESEARCH ARTICLE

Genetic Fidelity Testing Using SSR Marker Assay Confirms Trueness to Type of Micropropagated Coconut (*Cocos nucifera* L.) Plantlets Derived from Unfertilized Ovaries

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Abstract:

Background:

In vitro culture techniques provide an excellent platform for the multiplication of recalcitrant species such as coconut and thereby increase the homogeneity of the plantations. Clonal fidelity is one of the most important pre-requisites in a micropropagation protocol of crop species especially those with long life spans.

Objective:

The present study was conducted in order to determine the genetic homogeneity of coconut plantlets derived from unfertilized ovaries through somatic embryogenesis.

Method:

Twenty randomly selected plantlets at acclimatization stage, from two mother palms were subjected to Simple Sequence Repeats analysis. Thirteen highly polymorphic microsatellite primers were used for the detection of genetic fidelity in the clonal plantlets and their respective parent.

Results:

These plantlets showed no apparent differences among themselves and were comparable with the respective mother palm in the Simple Sequence Repeats analysis. The results obtained from this study suggest that there is no somaclonal variation or genetic instability occurring in plantlets that are regenerated from ovary explants.

Conclusion:

The absence of any sign of somaclonal variation suggests that somatic embryogenesis protocol did not induce the changes in gene structure, which had remained stable throughout the period that had been maintained *in vitro*. Determination of genetic fidelity of *in vitro* plants proved the suitability of regeneration protocol for large scale micropropagation applications for coconut.

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Keywords: Somatic embryogenesis, Somaclonal variation, Polymorphic, Microsatellite, Homogeneity, Micropropagation protocol.

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a versatile tropical plant which provides diverse and immense uses to the community and thus referred as 'tree of life'. It is predominantly a cross pollinated palm propagated through seeds and there is no viable option for a vegetative regeneration method. Production of homogeneous populations by seed propagation is not possible, resulting in great variability in economically important characters such as nut yield, kernel weight, oil content *etc* [1, 2]. *In vitro* culture techniques provide an excellent platform for the multiplication of recalcitrant species such as coconut and thereby increasing the homogeneity of the plantations. The interest in using tissue culture techniques for propagation of coconut has been significantly increased with the aim of developing a reliable clonal propagation method for both research purposes and commercial use. Recently, unfertilized ovary was identified as a more reliable explant for coconut micropropagation through somatic embryogenesis [3]. Even though the number of clonal plants obtained still remains low for commercial scale there is a green light with the continuous production of plantlets even at a low rate [4].

Clonal fidelity is one of the most important pre-requisites in a micropropagation protocol of crop species especially those having long life spans. The occurrence of genetic defects as a result of somaclonal variation in the sub clones of one parental line seriously limits the utilization of micropropagation systems both for other research uses as well for commercial practise. These variations are believed to be induced by the stress caused by tissue culture environment due to unusual culture conditions, frequent transfer of culture and influence of culture conditions like alterations in the supply of nutrients, different hormone concentrations and their ratios. These factors will cause DNA damage including microsatellite instability that is heritable thus challenges the exact clonal nature of the progenies.

Table 1. List of selected primers used in SSR analysis, primer sequence and the fragment size for each primer.

Oligo Name	Sequence	Fragment Size/Range (bp)	Annealing Temperature
CAC08 F	5'-ATC ACC CCA ATA CAA GGA CA-3'	188-210	56°C
R	5'-AAT TCT ATG GTC CAC CCA CA-3'		
CAC20 F	5'-CTC ATG AAC CAA ACG TTA GA-3'	124-133	54°C
R	5'-CAT CAT ATA CAT ACA TGC AAC A-3'		
CAC23 F	5'-TGA AAA CAA AAG ATA GAT GTC AG-3'	170-179	56°C
R	5'-GAA GAT GCT TTG ATA TGG AAC-3'		
CAC65 F	5'-GAA AAG GAT GTA ATA AGC TGG-3'	150-173	54°C
R	5'-TTT GTC CCC AAA TAT AGG TAG-3'		
CNZ04 F	5'-TAT ATG GGA TGC TTT AGT GGA-3'	130-166	53°C
R	5'-CAA ATC GAC AGA CAT CCT AAA-3'		
CNZ06 F	5'-ATA CTC ATC ATC ATA CGA CGC-3'	69-97	52°C
R	5'-CTC CCA CAA AAT CAT GTT ATT-3'		
CNZ10 F	5'-CCT ATT GCA CCT AAG CAA TTA-3'	108-152	56°C
R	5'-AAT GAT TTT CGA AGA GAG GTC-3'		
CNZ12 F	5'-TAG CTT CCT GAG ATA AGA TGC-3'		
R	5'-GAT CAT GGA ACG AAA ACA TTA-3'	218-229	54°C
CNZ21 F	5'-ATG TTT TAG CTT CAC CAT GAA-3'	220-250	54°C
R	5'- TCA AGT TCA AGT TCA AGA AGA CCT TTG -3'		
CNZ29 F	5'-TAA ATG GGT AAG TGT TTG TGC-3'	105-157	56°C
R	5'-CTG TCC TAT TTC CCT TTC ATT-3'		
CNZ40 F	5'-CTT GAT TGC TAT CTC AAA TGG-3'	143-155	56°C
R	5'-CTG AGA CCA AAT ACC ATG TGT-3'		
CNZ44 F	5'-CAT CAG TTC CAC TCT CAT TTC-3'	151-170	52°C
R	5'-CAA CAA AAG ACA TAG GTG GTC-3'		
CNZ46 F	5'-TTG GTT AGT ATA GCC ATG CAT-3'	101-120	56°C
R	5'-AAC CAT TTG TAG TAT ACC CCC-3'		

In the recent past, DNA based molecular markers have served as an important tool to assess the genetic homogeneity of micropropagated plants. Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and inter-retrotransposon amplified polymorphism (IRAP) have been used for screening

the genetic fidelity in micropropagated plants [5 - 9]. However, these methods involve the use of expensive enzymes and radioactive labelling. Also the techniques are laborious and not suitable for the routine application of genetic fidelity testing [10]. Microsatellite or simple sequence repeats (SSR) markers are short tandem repeats of two to five base pair motifs occurring throughout the eukaryotic genomes and are easily detectable *via* Polymerase Chain Reaction (PCR) [11]. Amongst the molecular markers, SSRs have been the most widely and extensively used in analyzing genetic stability of *in vitro* raised plants due to many desirable characteristics like co-dominant inheritance nature, high reproducibility, high allelic diversity, frequent abundance in organisms, and strong discriminatory power [11 - 14].

Although many reports are available on micropropagation of coconut only one report [15] exist on genetic stability of micropropagated coconut plants. However, this report does not compare the regenerants with their mother palms. This paper for the first time reports the true-to-type conformity of tissue cultured coconut plants derived from unfertilized ovaries not only among the progeny but also with the mother palm.

Table 2. Allelic distribution pattern of mother palm and respective clonal plantlets for each primer sequence.

SSR primer	DT Parent MDT and Clonal plantlets C1-C20	TSR Parent MTSR and Clonal plantlets C21 – C40
CNZ04	A1A2	A1A2
CNZ06	B1B3	B1B2
CNZ10	C1C3	C2C3
CNZ12	D1D2	D1D3
CNZ21	E1E2	E2E3
CNZ29	F1F2	F1F1
CNZ40	G1G3	G2G3
CNZ44	H2H4	H1H3
CNZ46	I1I2	I1I3
CAC08	J1J1	J1J2
CAC20	K1K2	K1K1
CAC23	L1L2	L2L2
CAC65	M1M2	M1M2

MATERIALS AND METHODS

Plant Material and Culture Conditions

In this study two coconut hybrids, CRIC65 (palm number DT-2) and CRISL98 (palm number TSR-12), were used to raise tissue cultured plantlets. CRIC65 is a cross between Sri Lanka Green Dwarf and Sri Lanka Tall coconuts while CRISL98 is a cross between Sri Lanka Tall and San Ramon Tall from the Philippines. Ovary derived plantlets were raised using the method described by Perera *et al.* [3], with slight modifications. Unfertilized ovaries were excised from female flowers of -4 stage (considering the most recently opened inflorescences as 0 stage, the next inflorescences to open was referred as -1 and inflorescence that would open in four months later was considered as -4 maturity stage) in adult coconut palms of the two hybrids. The basal part of the rachilla with the female flowers was separated from the inflorescence and disinfected with 2% (v/v) Clorox (5.25% active chlorine) for 15 minutes and rinsed with sterilized distilled water for five times under aseptic conditions. The ovaries (2 mm) were excised by removing sepals and petals under the stereo binocular microscope (Zeiss Stemi DV4).

The crushed explants were cultured in 15 mL of standard callus induction, CRI 72 medium [2] supplemented with 160 µM 2,4-dichlorophenoxycitic acid (2,4-D), 9 µM thidiazuron (TDZ), 0.1% (w/v) activated charcoal (Haycarb, Sri Lanka), 4% sucrose [3]. The pH of the medium was adjusted to 5.8 before solidifying with 0.25% (w/v) phytagel. The cultures were maintained in dark at 28 ^{o}C for 10 weeks without sub-culturing. Initial calli were further multiplied by dissection of embryogenic (ear like) structures Fig. (**1a**) under the stereo binocular microscope and subculturing into new callus induction medium (first cycle). After 6 weeks, the embryogenic structures that developed on callus from the first cycle were separated and subcultured in the same medium (second cycle). After two multiplication cycles, the embryogenic structures dissected from well-developed calli Fig. (**1b**) were sub-cultured into somatic embryo induction medium [CRI 72 medium containing 95 µM 2,4-D] for 4 weeks.

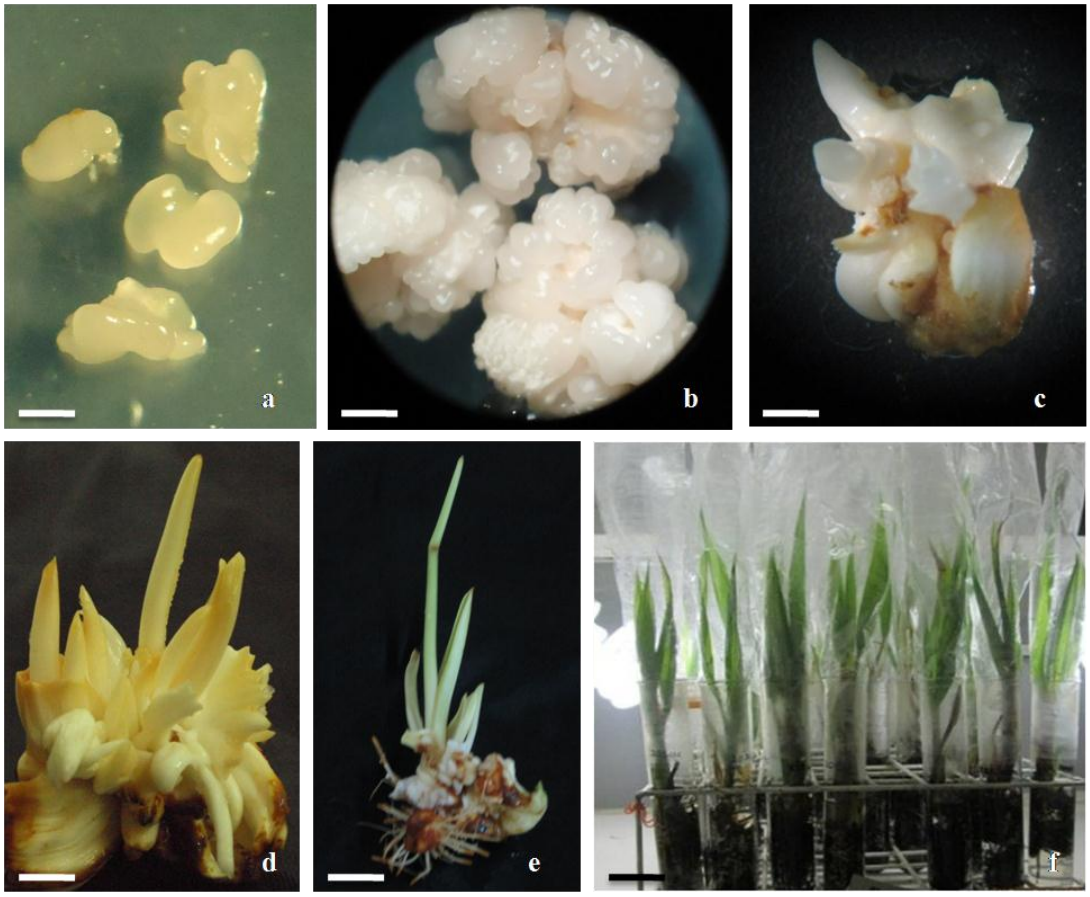

Fig. (1). Initiation of somatic embryogenesis and Plant regeneration from ovary explants of coconut (*Cocos nucifera* L.) **a** Ear like structures dissected from ovary derived callus (Bar = 2 mm) **b** ovary derived callus after second multiplication cycle (Bar = 5 mm) **c** Somatic embryos obtained after subculturing the calli onto somatic embryo maturation medium devoid of growth regulators (Bar = 5 mm) **d** Germinating somatic embryos with early shoot development stage (Bar = 5 mm) **e** Shoot and root development of the plantlet in conversion medium containing GA_3 (Bar = 1 cm)**f** Well developed complete plantlets ready to transfer into soil (Bar = 5 cm).

Calli that developed into shoot like structures were transferred directly to regeneration medium [modified Eeuwens Y_3 medium containing 20 µM of 2-isopentyladenine (2iP)]. The embryonic nodules from callus were sub cultured in to the somatic embryo maturation medium (modified Eeuwens Y_3 medium) for 4 weeks. The well-developed somatic embryos Fig. (**1c**) were transferred and maintained in regeneration medium (modified Eeuwens Y_3 with 20 µM 2iP) for four weeks. Germinating somatic embryos Fig. (**1d**) were transferred to modified Eeuwens Y_3 germination medium containing 0.45 µM Gibberelic acid (GA_3) to induce shoot formation. After shoot and root system is well developed Fig. (**1e**), the plants were transferred to a modified Eeuwens Y_3 liquid medium containing 0.45 µM GA_3 and maintained Fig. (**1f**) at 28 °C under 16 h photoperiod (PAR: 25 µmol m-2s-1), until transplanting into the soil.

Young leaves from twenty randomly selected *in vitro* raised clonal plants were collected carefully from each clone just before the plants were transferred to soil for the extraction of DNA. Spear leaves from mother palms of CRIC65, palm number DT-2 and CRISL98 palm number TSR-12 were collected from Bandirippuwa Estate, CRISL, Lunuwila, Sri Lanka, where these two palms are field planted.

DNA Extraction

Fresh leaf samples (100 mg each) were frozen in liquid N_2 and ground into a fine powder. The total genomic DNA was extracted using a PureLink® Genomic Plant DNA Purification Kit (Invitrogen) following the manufacturer's instructions. DNA was finally resuspended in 50 µL elution buffer and stored at –20 °C until further use. The purified total genomic DNA was electrophoresed on 0.8% Agarose gel and approximate quantification was done by comparing the fluorescent intensity with a series of known standard DNA solutions by staining with Ethidium bromide for band visualization.

Selection of SSR Primers

Thirteen SSR primer pairs were selected for the amplification of genomic DNA through PCR for the detection of genetic fidelity in the clonal plantlets and their respective parent, based on the previous genotypic information (Table **1**). The selected primers recorded the highest levels of polymorphism for coconut populations [16] including Sri Lanka Tall, Sri Lanka Green dwarf and San Ramon tall which are the three parents in the two coconut hybrids tested. The selected primers have been developed by Perera *et al.* [17], and Rivera *et al.* [18], and were purchased from Integrate DNA Technologies (IDT), Singapore (Table **1**).

PCR Amplification

PCR was performed in a 10 µL final volume comprising 30 ng genomic DNA and 2 µL of 01 x PCR buffer (GoTaq® flexi;Promega MADISON WI USA),1.5 mM $MgCl_2$ (Promega MADISON WI USA), 0.2 mM each of dNTPs, 1U of Taq DNA polymerase (5u/µL: GoTaq® flexi;Promega MADISON WI USA), 1.0 µM each of forward and reverse primer (10 µM). Amplifications were carried out in thermal cycler (BIO-RAD; MyCycler™) programmed at 94 °C for 5 min for initial denaturation, followed by 94 °C for 30 sec, annealing temperature depending on the primer used (Table **1**). for 30 sec and 72 °C for 1 min for 35 cycles followed by a final step of extension at 72 °C for 5 min. Final holding temperature was 4 ^{o}C.

SSR Marker Analysis

PCR samples were electrophoresed on 6% (w/v) polyacrylamide gel and bands were visualized by silver staining. Plantlets regenerated from *in vitro* cultured unfertilized ovaries were tested by SSR marker analysis for genetic integrity. The banding patterns of the samples were scored and compared with respective mother palms.

RESULTS AND DISCUSSION

At present, CRISL is able to regenerate clonal coconut plants from unfertilized ovaries at considerable quantities [4]. Before any of the tissue culture protocols are being implemented as a commercial practice, the protocol should be tested for any genetic disorders especially for long life species.

Microsatellite or SSR markers are particularly suitable for detecting somaclonal variations in micropropagated plants as repeated DNA sequences are known to show increased levels of instability in tissue culture (Parida *et al.,* 2009) [19]. The thirteen primers used in the present study were all informative and generated amplicons for the detection of the genetic fidelity of the progeny. Amplification pattern of ovary derived clonal plants of several SSR primers is shown in Figs. (**2a** to **2e**). Out of 96 plants regenerated from TSR-12 mother palm and 88 plants regenerated from DT-2 mother palm, twenty randomly selected plantlets from each mother palm at acclimatization stage were subjected to SSR analysis. These plantlets showed no apparent differences in vegetative growth among themselves. Each tested SSR primer pair produced clear reproducible bands ranging in size from 50-240 bp and all the clonal plantlets scored identical alleles with the respective mother palm at all SSR loci analysed in the current study (Fig. **2** and Table **2**).

At each of the SSR marker locus two alleles were scored and they were visualized as two bands when different alleles had been donated by each parent, (*i.e.* Sri Lanka Green Dwarf and Sri Lanka Tall in the hybrid DT and Sri Lanka Tall and San Ramon in the hybrid TSR) and the two alleles were visualized as a single band when the two parents donated similar alleles to the hybrid. Marker loci CNZ04 and CAC65 scored similar alleles for the two hybrids. DT parent and all the clonal plantlets derived from that parent scored heterozygous genotypes at all the tested SSR marker loci. Homozygous alleles were scored for the TSR parent and its clonal progeny at marker loci CNZ29, CAC20 and CAC23 indicating similar alleles for Sri Lanka Tall and San Ramon while heterozygosity was observed at the rest of the 10 marker loci for TSR parent and the clonal progeny separating the Sri Lanka Tall and the San Ramon coconut varieties. Sri Lanka Tall is a common parent in both the hybrids. However, the two hybrids have not been derived from the same Sri Lanka Tall palm and hence this allele may or may not be similar at different loci owing to the heterozygous individuals and heterogeneous populations of Sri Lanka Tall coconuts. Common Sri Lanka Tall alleles were scored at all the marker loci except at CNZ44 where the two different parent of Sri Lanka Tall were different in the two parents.

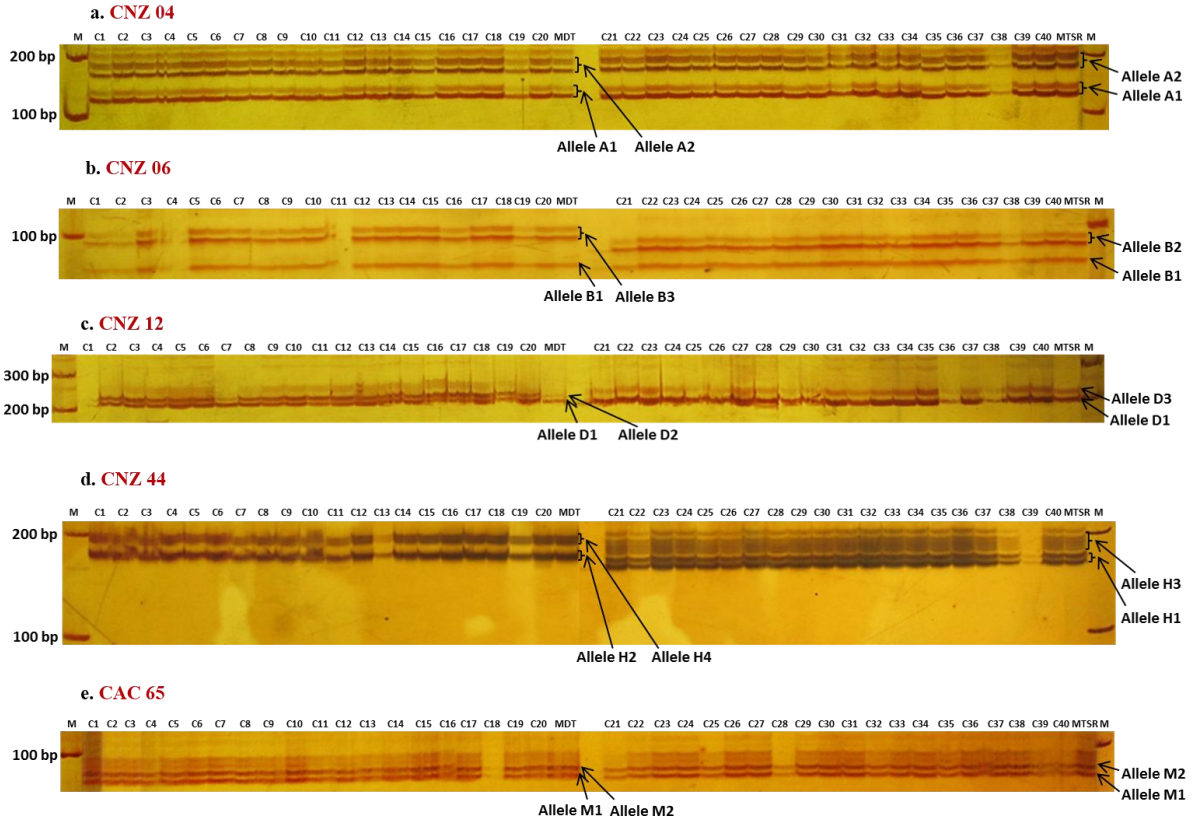

Fig. (2). Amplification of DNA extracted from plantlets derived from unfertilized ovaries of two mother palms with SSR primers (a) CNZ04, (b) CNZ06 (c) CNZ12 (d) CNZ44 primers and CNZ65. Lanes: M 100 bp DNA ladder, MDT: DT-2 mother palm, MTSR: TSR-12 mother palm, C1-C20 clonal plants raised from DT-2 mother palm and C21- C40 clonal plants raised from TSR-12 mother palm.

This is the first report of the true-to-type conformity of tissue cultured coconut plants derived from unfertilized ovaries with the respective mother palm. Previously, only Fernando *et al.* [15], have reported the assessment of genetic stability of tissue culture-raised plants of coconut using SSR markers but it failed to compare the plantlets with their respective mother palms. Visual observations of polyacrylamide gel images proved the genetic equality among the *in vitro* plantlets. When the DNA banding patterns of the plantlets were compared with each of the mother palm, there was no variation between the mother palm and the resulting *in vitro* clonal plantlets at any of the tested marker loci. Consequently, all the *in vitro* propagated clonal plantlets were proved to be genetically equal to the respective mother palm providing no evidence for somaclonal variations *in-vitro*. The results are in line with the previous reports on the detection of genetic fidelity in micropropagated plants using SSR markers in other plant species including *Saccharum officinarum* [20], *Populus termuloides* [21], *Olea maderensis Olea europaea* [22] and *Psidium guajava* [23].

The general belief is that multiplication involving a callus phase is more vulnerable for DNA damage during micropropagation [24, 25]. For an example; in oil palm (*Elaeis guineensis* Jacq), 5% of somatic embryo-derived palms showed an abnormality that is called the ‘mantled’ phenotype [26]. This is claimed to be a somaclonal variation resulting in a conversion of the male floral organs into supernumerary carpels [27] and may lead to partial or complete flower sterility, depending on the severity of the abnormality [26]. It has been reported that hypo-methylation during tissue culture contributes to the origin of this ‘mantled’ phenotype in tissue culture effectively preventing the clonal propagation from elite palms [28]. In our study, the coconut plantlets were derived through somatic embryogensis and a prolonged callus phase was introduced by multiplying callus in two additional cycles. Even though the plants have been growing for more than two and a half years under *in vitro* conditions with different hormone and chemical levels, the present analysis showed no evidence for genetic variations or incidences of mutants among the plantlets, proving the non-inducement of any genetic variation in the clonal plantlets during the micropropagation protocol. The most common event that triggers the tissue culture induced variation is due to the cell cycle disturbance caused by the exogenous hormones applied in the culture process [29]. The present study indicates that the level of hormones

especially 2, 4-D and BAP used for coconut plant regeneration is good enough to retain the genetic integrity of regenerated plants indicating the above hormone levels not to be deleterious to genetic integrity of coconut cultures even though the protocol is developed through a callus phase. High genetic and epigenetic stability has been reported in coffee (*Coffea Arabica*) plants derived from embryogenic suspensions [30]. It has also been reported that long term culture and cell culture ageing in coffee, resulting in high rates of mutations and chromosomal re-arrangements which are directly linked to somaclonal variations (31). Later Etinne *et al* [32] reviewed that, the genetic and epigenetic alterations were particularly limited during somatic embryogenesis in coffee but that the main change in most of phenotypic variants were the results of aneuploidy showing that mitotic aberrations are the main cause for somaclonal variations. Even though SSR are reported to be a good tool to detect genetic changes, certain changes such as, epigenetic changes due to genome methylation or transposable elements and mitotic changes like aneuploidy could also occurred during somatic embryogenesis and not be detected by SSR as explained. Nonetheless, genetically uniform plantlets detected by SSR analysis in the present study is a positive move towards the clonal multiplication of improved genotypes (CRIC65 and CRISL98) and should be extended to other recommended cultivars also.

Being hybrids, both CRIC65 and CRISL98 are heterozygous receiving one set of chromosomes from each of the two of their respective male and female parents. The heterozygosity of the two hybrids was confirmed with the visualization of two bands at each of the SSR marker locus tested. This observation was made possible due to the co-dominant inheritance of the microsatellite marker system. In addition to the heterozygosity of the hybrid mother palms, the clonal progeny was shown to be hybrids giving evidence for the true to type *in vitro* propagation with the explant unfertilized ovary.

Identical alleles were scored at the SSR loci CNZ04 and CAC65 for Sri Lanka Green Dwarf and San Ramon coconut varieties. Sri Lankan Green Dwarf is a dwarf coconut variety while San Ramon is an exotic coconut variety introduced from the Philippines. The coconut variety Sri Lanka tall resembles Indo-Atlantic coconuts while dwarfs including King coconut in Sri Lanka and San Ramon resemble the far-east/Pacific coconuts [17, 33]. Furthermore, it is suggested that the dwarf population is a subset of the main tall population, and has evolved directly from tall coconut. That means the 'dwarf' originated from earliest 'tall' coconuts and maintained most of its original genome because of its autogamous behaviour [17, 33].

A study of Perera *et al.* [17], reported that some alleles in Sri Lankan dwarfs are completely absent in Sri Lankan Tall, suggesting that dwarf coconuts in Sri Lanka represent a separate introduction, rather than having evolved from Sri Lankan tall coconuts. This is further saying that no Sri Lankan dwarfs are found in the same group as Sri Lankan Tall [17]. Moreover, dwarf coconuts are of similar stature and fruit features irrespective of the geographical location. However, 'tall' genome has undergone many changes due to bottle-neck effects of selection, though it had retained the tall stature and fruit characteristics irrespective of its dispersion from Far-East to Indo-Atlantic regions across Africa [34]. The results of the current study are in accordance with these earlier findings as has been demonstrated at the SSR loci CNZ04 and CAC65. Accordingly, while these SSR marker loci are useful to differentiate Sri Lanka tall San Ramon they are not informative to distinguish Sri Lankan Green Dwarf and San Ramon.

Hybrid coconuts mainly between the tall and dwarf coconuts are genetically improved and are recommended for planting in many of the coconut growing countries. The propagules of hybrid coconuts are seed nuts and they are mass produced at isolated seed gardens where the relevant parents have been grown. This method of production of hybrid planting material is practically difficult and very much labour intensive. Feasibility of the *in vitro* culture vegetative propagation is a much desired future goal in the production of planting material of the improved coconut cultivars. The genetic fidelity of such material is of primary importance and the current study is a progressive and encouraging step towards a viable *in vitro* culture based vegetative propagation method for coconut.

CONCLUSION

Assessment of genetic fidelity of micropropagated plants at an early stage helps fine tuning of protocol parameters and judges the suitability of regeneration protocol for large scale applications. The genetic stability of unfertilized ovary tissue raised plantlets was tested by SSR markers. The banding pattern of PCR amplified products from plantlets regenerated through somatic embryogenesis was monomorphic across all the micropropagated plantlets and was identical with their respective mother palms. Thus for the first time, SSR profiling confirmed the genetic purity, of coconut plantlets derived *in vitro* using unfertilized ovaries. Present study is a good start to test the genetic fidelity of the coconut plantlets regenerated by SE process but further studies must to be done at the field level to verify it.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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