DNA fingerprinting to distinguish the coconut type, San Ramon

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Abstract

San Ramon, an introduced type of coconut from Philippines, has long been in Sri Lanka. San Ramon has the advantage of producing high copra, high oil, and high-quality timber and also seemed to be less variable for bunch characters. In addition, it is a type of coconut having an ability to withstand long droughts and also coconut mites. Due to these outstanding beneficial characters, San Ramon has been used as a parent for effecting various crosses to transmit the drought tolerance nature and high copra outturn, to the offspring. The physical structure of San Ramon palms is much comparable to tall type coconuts though there are some specific characters predominantly seen in favourable environments. On top of these circumstances, it is not easy to separate San Ramon from other Talls and virtually impossible to distinguish pure San Ramon from San Ramon crosses by means of only morphological markers. Therefore, a molecular finger-printing method was attempted to distinguish pure San Ramon using already developed 10 coconut SSR primers. The results indicate the possibility of distinguishing pure San Ramon from its crosses with the help of 2 coconut SSR primers, CNZ6 and CNZ44.

Key words: Coconut, DNA fingerprinting, molecular marker, San Ramon, SSR primers.

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Introduction

DNA fingerprinting of plants or plant genotyping is a technology that has been established and is primed for very widespread practical applications such as, identification of plants in commerce, plant breeding and research. Our interest as plant breeders and molecular biologists is to identify plants accurately to use in crossing programmes and also to test hybridity. Plant breeding applications range from marker assisted selection (MAS) to the confirmation of identity of parents and progeny in breeding populations.

The coconut (*Cocos nucifera*) is called the tree of life because it provides humans with such a wide range of essential products; food and drink (coconut milk, coconut cream, desiccated coconut, tender coconut water, coconut arrack, coconut vinegar, coconut honey and coconut jaggery); materials for housing, fuel and many industrial uses such as shell charcoal, activated carbon, shell powder, shell handicrafts, furniture, coir products, soaps, and cosmetic industries etc. It can be taken as a treasure for people in many tropical countries considering it as a caloric supplier, foreign exchange earner and also a crop with a multitude of uses for income generation for poor farmers. It plays a unique role in the diets of mankind because it is the source of important physiologically functional components found in the fat part of whole coconut and of desiccated coconut as well as in the extracted coconut oil. The medicinal uses of coconuts, anti-bacterial, anti-viral and anti-fungal activities due to lauric acid and capric acid, are many and varied (Enig, 1999; Dayrit, 2000). Also it has been shown that natural coconut fat in the diet leads to a normalization of body lipids, protects against alcohol damage to the liver and improves the immune system's anti-inflammatory response (Enig, 2001).

San Ramon, an introduced type of coconut from Philippines, has long been in Sri Lanka (Fernando, 1987). San Ramon nuts are very large and round with a thick kernel and the underside of the dehusked nuts is flat. The physical structure of San Ramon palms is much alike to tall type coconuts. Inherently, they are huge erect tall trees with stout trunks and massive boles under a favourable environment. But the above description of the physical structure of the palm could be changed under different soil and climatic conditions. Therefore, it is not very easy to separate San Ramon from Tall and totally impossible to distinguish pure San Ramon from San Ramon crosses.

San Ramon has the advantage of producing high copra with a low cost of production since it has high copra content of 350-400 g per nut while in other coconut varieties it varies from 210-225 g (Fernando, 1987). San Ramon inherits the potential of producing 51% more copra per nut than Sri Lanka Tall palms and 50% more copra per nut than the improved tall, CRIC60. Preliminary investigations have shown that the oil extraction efficiency of copra is much higher than Sri Lanka Tall (Fernando, 1999). Another special feature of San Ramon is its drought tolerance. The extent of stress tolerance in San Ramon was investigated by Ranasinghe (1989) and reported that the levels of total soluble sugars, starch content and proline content were significantly higher both in San Ramon and its crosses. Accumulation of proline under water deficit conditions has been shown to be a sensitive indicator of drought tolerance in annual crops (Singh *et al*., 1973), and Jayasekara *et al*., (1983) confirmed this phenomenon for coconut since higher concentrations of proline accumulation was observed during dry conditions for genotypes identified as stable when assessed for drought sensitive physiological parameters.

In addition to that, it seemed to be less mite prone type of coconut. A study conducted by Palomar *et al*. (1994) on the wood quality of San Ramon timber has shown that high values in all the mechanical properties relevant to timber quality. The form San Ramon is believed to be originated from Mindanao islands in the Philippines where frequent fluctuations in climatic conditions are experienced and as a result the cultivar may have accumulated favourable genes over a long period of adaptation (Santos *et al*., 1984). In a study

carried out on 7 tall populations in the Philippines, Balingasa and Carpio (1983) reported that San Ramon had the lowest coefficient of variability for bunch characteristics. Having these outstanding beneficial characters, San Ramon has been used and also is being currently used for various crosses to transmit the drought tolerance nature and high copra outturn to the offspring.

A useful molecular marker technique in identification of different coconut types and their crosses should be based on a method that can be reliably reproduced in any laboratory. Out of many molecular marker methods, microsatellites (Simple Sequence Repeats or SSRs) (Powell *et al*., 1996) has been accepted as an identical tool for genotyping individuals and hybridity testing, particularly because they are reproducible and co-dominant in nature with an excellent ability in differentiating homozygotes from heterozygotes, in addition to its highly polymorphic and multi-allelic nature coupled with simple to perform PCR based approach. However, the use of SSR is limited to a small number of crop species as the development of these markers for each crop species is too expensive. Despite this difficulty, substantial number of SSR primers for the PCR has been developed for coconut (Perera *et al*., 1999; Rivera *et al*., 1999; Teulat *et al*., 2000).

The only available coconuts in Sri Lanka are under two main varieties, Tall and Dwarf apart from the introduced type, San Ramon. Consequently, until now the crosses were made within those three types of coconut. The first introduction of San Ramon to Sri Lanka was made by a private grower and was planted at the Clovis Estate, North Western Province of Sri Lanka (CRI, 1987). A few seedlings arising from open pollinations from the Clovis introduction was planted at the Coconut Research Institute of Sri Lanka (CRISL) and these palms were used as parents to raise selfed progenies. Therefore, some palms, currently available in CRISL and also believed to be San Ramon, might not be pure San Ramon due to initially introduced open pollinated seedlings from Clovis. Under this confused state, it is worth to develop a DNA fingerprinting method to distinguish San Ramon from its crosses and also from Tall and Dwarf types to verify the identity of parents and progeny in breeding populations in the absence of clear morphological differences among them. In addition, misidentification of varieties in long term breeding trials may pose serious problems. Therefore, this study was carried out with an objective of developing a molecular finger printing method using already developed coconut SSR to distinguish pure San Ramon (SR) from Tall x San Ramon (TxSR) and Dwarf Green x San Ramon (DGxSR).

Materials and methods

Genetic material

A total of 77 individuals representing 3 coconut varieties, Tall, Dwarf and San Ramon (each variety was represented by 5 individuals) and 62 palms believed to be San Ramon were used in this study. DNA was extracted from each individual separately according to Doyle and Doyle (1990). Diluted DNA was quantified using standard λ DNA in agarose gels. The DNA was further diluted to 10 ng/µl as working samples for microsatellite primers.

Microsatellite (SSR) primers

Ten coconut SSR primers, which had been identified as suitable primers to clearly distinguish Tall and Dwarf Green coconuts (Bandaranayake, 2002), tested on selected genetic material. All information on selected SSR primers is given in Table 1.

PCR and gel electrophoresis

PCR was carried out with each primer pair of 10 µl reactions, which contain, 10 ng genomic DNA, 10 µM Forward primer, 10 µM Reverse primer, 0.2 mM deoxyribonucleotides, 1.5 mM magnesium chloride, 1 x PCR buffer and 1 unit of *Taq* DNA polymerase. Standard cycling conditions were: denature at 94°C for 1 min, annealing at optimum temperature for 1 min, extend at 72°C for 2 min and repeat this for 30 cycles.

Polyacrylamide gel electrophoresis (PAGE) was used since it provided greater resolution than agarose gel electrophoresis in the analysis of closer alleles. Silver staining procedure (Bassam and Caetano-Anolles, 1993) was used for staining the polyacrylamide gels and alleles were scored under an UV transilluminator.

Results and discussion

At first, DNA of individual palms of 3 varieties was amplified with all 10 SSR primer pairs. All 5 individual palms under each variety were given the same alleles under each SSR primer except one individual of San Ramon. As a result, that individual San Ramon was discarded from our experiment and other individuals were bulked under each variety. These 3 bulks, Tall, Dwarf and San Ramon, were then used to represent each variety under 10 selected SSR primer pairs.

Of the 10 SSR primers used, only three, viz; CAC8, CNZ6 and CNZ44, showed clear polymorphic bands between the three varieties of coconut, Tall (a_1), Dwarf (a_2) and San Ramon (a_3 or a_3 and a_4) (Table 2). These three SSR primers were then used to amplify the DNA of other palms, which were supposed to be San Ramon, and three pure varieties. Out of 62 palms, which were supposed to be San Ramon, only 52 showed either one or both San Ramon specific alleles as a_3 or a_4 or a_3 and a_4 under CAC8.

With CNZ6, only 27 individuals showed a San Ramon specific band, a_3. Figure 1 illustrates a sample of 19 individuals who were supposed to be San Ramon with 3 pure varieties [Tall (T), Dwarf (D) and San Ramon (SR)] on either side of the gel. The 3 alleles under Tall, Dwarf and San Ramon are much apart under CNZ6 and therefore, the scoring was very easy. It shows that 15 individuals out of 19 are SR except sample numbers 1, 2, 3 and 4. Sample numbers 1, 2 and 4 could be considered as DxSR hybrids while sample number 3 has not worked. These results indicate that the SSR primer CNZ6 can be successfully used to distinguish Tall, Dwarf and San Ramon having presence of alleles a_1, a_2 and a_3 respectively. The presence of both a_2 and a_3 alleles in heterozygous state confirms the status of DxSR hybrid.

Only 35 individuals showed San Ramon specific allele a_3 in CNZ44 with a small shift in the size of the bands under 3 varieties. Figure 2 clearly demonstrates a gel showing the banding profile of a sample of individuals with CNZ44. It shows that 15 individuals out of 19 as SR, except the sample numbers 7 and 14 are TxSR hybrids while numbers 3 and 12 have not worked. Since the primer CNZ44 produces an exclusive band for SR (a_3) and different bands for Tall and Dwarf (a_1 and a_2 respectively), sample numbers 7 and 14 could be confirmed as TxSR hybrids with the presence of both a_1 and a_3 bands. When we consider SSR primers CAC8 and CNZ6, all 27 individuals, which have shown San Ramon specific allele under CNZ6, were available under CAC8. Only 24 individuals out of 27, which have shown San Ramon specific alleles under CNZ6, were present under CNZ44. Considering CAC8 and CNZ44, the same 24 individuals have come up as pure San Ramon. Results are summarized in Table 3.

In view of a single primer, CAC8 has shown many individuals (52 out of 62) as San Ramon. However, CAC8 was not a reliable primer to score San Ramon since it has given 2 alleles for San Ramon and the presence of those 2 alleles was not consistent because some individuals have one allele, some have the other allele and some have both alleles. Accordingly, the individuals who had either one allele or both were scored as San Ramon. Therefore, the scoring was not too consistent.

Considering the primer pairs at a time, CAC8 and CNZ6, individuals identified as San Ramon under CNZ6 was a sub group under CAC8. In a similar way, the palms established as San Ramon with CNZ44 was a sub group with CAC8. Therefore, CAC8 could be excluded from this analysis since it was not an

Table 1. Selected 10 SSR markers, their repeat motifs, annealing temperatures, and estimated fragment sizes according to Perera *et al*., (1999), Rivera *et al*., (1999) and Teulat *et al*., (2000)

Locus	Repeat motif	Annealing temperature (^{0}C)	Fragment size (bp)
CAC2	$(CA)_{12}(AG)_{14}$	58	220
CAC6	$(AG)_{14}(CA)_9$	58	158
CAC8	$(AG)_{10}(CA)_9$	58	190
CAC10	$(TA)_6CATA(CA)_{11}(TA)_8$	58	198
CAC13	$(CA)_9(TA)_5A(TA)_4(CA)_6$	58	160
CAC23	$(CA)_8$	54	192
CNZ6	$(CT)_{15}$	53	85
CNZ29	$(GT)_{22}(GA)_2CA(GA)_{11}$	51	135
CNZ40	$(CT)_{20}$	53	151
CNZ44	$(GA)_{15}$	53	165

Table 2. Ten selected primers and their alleles under each variety

SSR primer	Alleles under each variety (Different alleles were marked as a_1, a_2, a_3 and a_4 under each SSR primer)		
	Tall	Dwarf	San Ramon
CAC2	a_1	a_2	a_2
CAC6	a_1	a_2	a_2
CAC8	a_1	a_2	a_3 , a_4
CAC10	a_1 , a_2	a_3	a_3
CAC13	a_1 , a_2	a_2	a_1 , a_2
CAC23	a_1	a_2	a_2
CNZ6	a_1	a_2	a_3
CNZ29	a_1 , a_2	a_2	a_1 , a_2
CNZ40	a_1	a_2	a_2
CNZ44	a_1	a_2	a_3

Table 3. Number of individuals identified came up as San Ramon under a single primer and combinations of primers

CAC8	CNZ6	CNZ44
52	27	35
27		24
24		

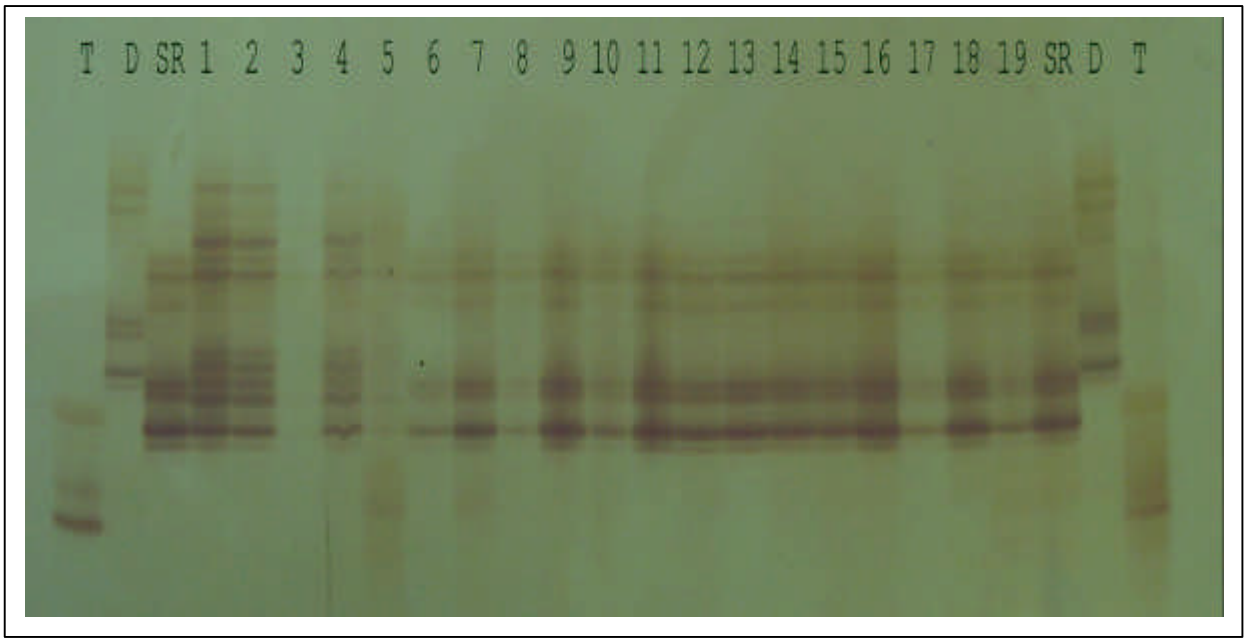

Figure 1. SSR banding profile of Tall (T), Dwarf (D), San Ramon (SR) with some individuals who supposed to be SR (from 1-19) with primer CNZ06

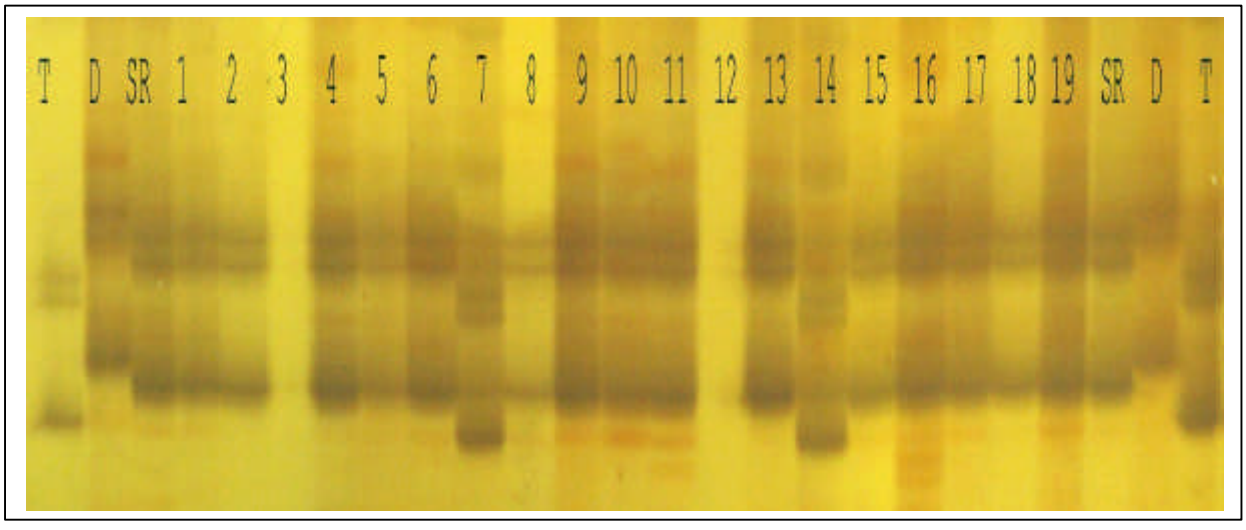

Figure 2. SSR banding profile of Tall (T), Dwarf (D), San Ramon (SR) with some individuals who supposed to be SR (from 1-19) with primer CNZ44

appropriate primer to spot pure San Ramon. Thus, the results indicate the possibility of using 2 SSR primers, CNZ6 and CNZ44, as molecular markers to distinguish pure San Ramon from its crosses.

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