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Analysis of segregating populations of West Coast Tall variety of coconut using microsatellite markers

R. Manimekalai*, V. B. Rubavathi, Regi J. Thomas[1], R.V. Nair and M.K. Rajesh

Central Plantation Crops Research Institute, Kasaragod - 671 124, Kerala, India

[1]Central Plantation Crops Research Institute, Regional Station, Kayangulam, Kerala, India

Abstract

In vitro antagonistic studies with native Trichoderma spp viz., T.viride, T.harzianum & T.hamatum and a bacterial strain of Pseudomonas fluorescens revealed that the mycelial growth of the coconut pathogens viz., Ganoderma applanatum and G.lucidum (basal stem rot pathogens) and Thielaviopsis paradoxa (stem bleeding pathogen) was highly inhibited by T.viride (more then 80%). Pseudomonas fluorescens completely inhibited the growth of both the species of Ganoderma, whereas only 33% inhibition was observed in the growth of T.paradoxa. All the three species of Trichoderma were found to be very effective in producing specific volatile metabolites against T.paradoxa. The volatile metabolites of P.fluorescens completely inhibited G.applanatum and G.lucidum but non-volatile metabolites were not effective. Application of 50 g of talc formulation of Trichoderma viride or T.harzianum or T.hamatum with combination of 5 kg neem cake/palm/year in basins around the palm was found to be effective in the management of basal stem rot disease and smearing of talc powder paste of Trichoderma viride or T.harzianum or T.hamatum reduced bleeding patches due to stem bleeding disease. Developed a common biointensive IDM package against basal stem rot and stem bleeding diseases.

Key words : Coconut, microsatellite, mapping, selfed progenies

Introduction

Novel genetic tools make use of several molecular marker techniques, which permits establishment of linkage maps in crop plants In addition, these linkage maps allow breeders to conduct applied research to identify, characterize, and use genetic variability in plants. Most linkage maps in plants have been obtained from segregating populations derived from crosses between inbred lines. Such populations or the required number of two-generation pedigrees are generally not available in coconut and are difficult to obtain due to significant space and time constraints. In coconut, genetic linkage maps could prove a very powerful tool for accelerating breeding processes. In view of the time consuming process in coconut breeding, modern molecular technologies like DNA markers have a great potential to assist in and accelerate breeding programmes. Various DNA markers have been applied to coconut for genetic diversity and DNA fingerprinting (Lebrun *et al.*, 1998; Perera *et al.*, 1999; Meerow *et al.*, 2003; Manimekalai and Nagarajan, 2006). Among the molecular markers, microsatellite markers are considered to be the best for mapping studies. Microsatellites are highly polymorphic even between closely related individuals within a species due to the number and frequency of alleles detected (Edwards *et al.*, 1996). Microsatellite markers are co-dominant and are inherited in a simple Mendelian fashion (Bruford and Wayne, 1993). Microsatellite regions occur frequently in the genome (Akkaya *et al.*, 1992).

In coconut linkage map was constructed based on alternative methodologies like use of F_1 segregating populations. The first linkage map on coconut was reported by Rohde *et al.* (1999) using 52 F_1 plants from the MYD 20 C LAGT 07 using ISTR. First linkage map of 16 linkage groups for a coconut type Rennell Island

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Scientist (Biotechnology), Central Plantation Crops Research Institute, Kasaragod -671 124, Kerala

Tall (RIT) was constructed by Lebrun *et al.* (2001), using SSR DNA markers. They scored Polymorphic DNA fragments for presence and absence in parents and F_1 progenies. They Herran *et al.* (2000) constructed a linkage map in coconut for the two parents of the cross MYD (Malayan Yellow Dwarf) X LAGT (Laguna Tall). In heterozygous crops due to presence of heterozygous loci, the progenies will segregate on self pollination. So the selfed population itself used for the linkage map construction in many cross pollinated crops (Grivet *et al.*, 1995). The same strategy is attempted in coconut; and with the use of microsatellite markers the progenies are evaluated as 1:1 segregation for homozygotes and heterozygotes in S_1 generation. The present paper deals with the microsatellie marker characterization of selfed progenies of a heterozygous tall mother palm with a view to construct linkage map in coconut.

Materials and Methods

DNA extraction

The leaf samples were collected from mother palm (WCT, Palm No.418). This palm is located in a farmer's plot in the Kottayam district of Kerala where more than 90 % of the palm is highly diseased due to root (wilt). This palm is aged more than 65 years and is high yielding and disease free. Female flowers produced in this palm were selfed and selfed seed nuts were harvested and grown in the nursery. Leaf samples from 53 seedlings (sprouted) and the mother palm were taken for molecular studies. Modified SDS method was adopted for DNA extraction (Upadhyay *et al.*, 1999).

Two gram of leaf material was frozen in liquid nitrogen and ground to powder in a pestle and mortar. 2.5% of poly vinyl (poly) pyrrolidone (PVPP, MW 40000) was added to the ground powder, mixed well and transferred to a centrifuge tube containing 10 ml of pre heated extraction buffer (150 mM Tris pH 8.0; 1.5 M NaCl; 1 % SDS). The mixture was incubated at 65 °C for one hour with intermittent mixing. Equal volume of Chloroform: Isoamyl alcohol mixture (24:1 V/V) was added and homogenized by gentle inversion for 15 min and centrifuged at 12,500 rpm for 15 min. at room temperature. The clear aqueous phase was then transferred into equal volume of ice-cold isopropanol and mixed by gentle inversion. The DNA spool was collected in 1.50 ml microfuge tubes and air dried. 500 ml of TE was added to dissolve the DNA and then 3 ml of (10 m g / ml) RNase was added and incubated at 37 °C for 30 min. To this equal volume of Phenol: Chloroform: IAA was added, mixed well and centrifuged at 14000 rpm for 15 min. The aqueous phase was transferred to 1.5 ml microfuge tube. Then 2.5 volume of absolute Alcohol and 1/10 volume of 3 M Sodium acetate were added and incubated at 4 °C for 1 hour. It was centrifuged at (14000 rpm for 10 min.) and the supernatant was discarded. To the pellet, 500 ml of 70 % ethanol was added to wash the DNA pellet by centrifugation. Alcohol was discarded and DNA pellet was air dried completely and dissolved in 100 ml of TE and stored at 4°C.

Quantification

Quantification of DNA was done using spectrophotometer and the purity was checked by running in 0.8 % agarose gel.

PCR amplification and denaturing polyacrylamide gel electrophoresis

DNA amplification was performed in a 10 μl volume with primer concentration of 0.2 μm each of forward and reverse primer. The PCR product was separated in 7.5% polyacrylamide gel and the segregated alleles were visualized through silver staining. The alleles were determined and the data was entered in MS Excel. The Segregation ratio, recombination frequency and LOD Score were calculated as described by Lorieux *et al.* (2000) using MAPDISTO software version 1.4.3 The recombination frequencies converted to map distance using Kosambi map function (Kosambi, 1944).

Linkage Analysis

In linkage analysis, the co-dominant nature of mother palms was screened with different primers. These primers were used earlier by Perera *et al.*(1999) and Meerow *et al.* (2003). Out of fifty primers screened, ten primers showed the co-dominant nature of loci. Out of ten primers, eight were used for the amplification of the DNA from 53 seedlings derived by selfing the mother palm.

Results and Discussion

The DNA samples were isolated from selfed coconut progenies of WCT 418. In the present study, coconut specific microsatellite primers, CnCirB3, CnCirC11, CNZ01, CNZ02, CNZA4, CNZ18, CN11E10, CNZ40 were used and the details are furnished in table 1. All the eight primers produced unambiguous marker and were reproducible. Fig. 1 shows the SSR profile of some of the samples. However, they have also been shown to segregate as dominant markers in some case. It might be attributed to segregation distortion (Sankar and Moore, 2001). Out of Eight loci, CNZA4 and CNZ40 showed 1:1 segregation, here SSR markers segregated as co dominant markers.

Map of declared loci in linkage map

In linkage studies, the most important parameter involved is recombination fraction, the "r" value.

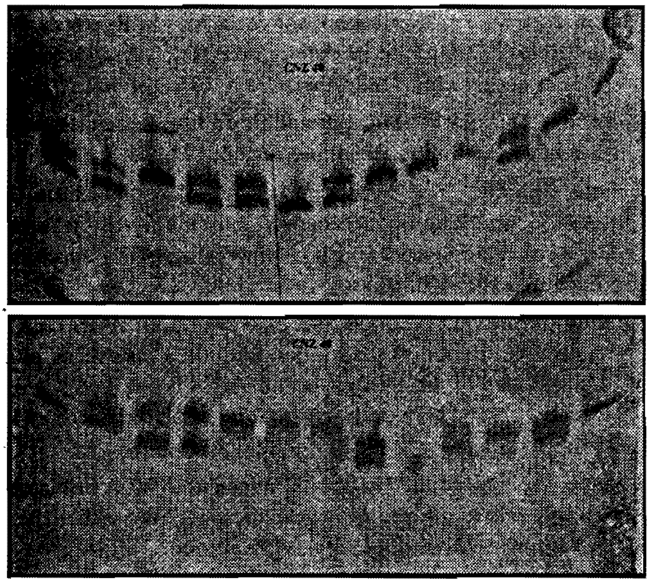

Fig. 1. SSR Marker profile of selfed coconut progenies produced by the primer C NZ40

Recombination fractions, are estimated from the proportion of recombinant gametes. Information about the gamete that was received by an offspring depends on the genotypes of the offspring.

The map of eight loci showed that the "r" value was minimum (r=0.382) between CNZ A4 and CNZ40 and maximum between by CNZ02 and CnCirC11 (r=0.750). The details are given in Table 2.

The LOD scores

To test whether or not two loci are really linked, the statistical test to perform is the likelihood versus a certain recombination fraction (r). Likelihood is a function of the recombination fraction r, and the likelihood subscripts of different models are compared by calculating the likelihood ratio. The LOD scores showed maximum value between the locus CnCirC11 and CNZ02 (1.36), and the minimum was between CN11E10 and CNZ180. LOD score of 1 to 2 is interesting, 2 to 3 is suggestive and greater than 3 is the proof of linkage. Here all the locus showed LOD score less than 3, indicating no linkage between them (Table 2).

Table 2. Map of declared loci in linkage map

	Locus	r	d	LOD
1	CnCirB3			
		0.615	172.67	0.30
2	CnCirC11			
		0.750	172.67	1.36
3	CNZ02			
		0.621	172.67	0.37
4	CN11E10			
		0.500	172.67	0.00
5	CNZ18			
		0.630	172.67	0.40
6	CNZ01			
		0.731	172.67	1.25
7	CNZA4			
		0.382	50.37	0.41
8	CNZ40			

r-Recombination fractions, d-Map distance, LOD-Logarithmic Odds score

Chi square (χ^2) Segregation of all declared loci

Based on Chi square test, 1:1 segregation was observed for the locus CNZA4 and CNZ40. As the mapping population was evaluated as test cross, 1:1 segregation was observed. The 1:1 segregation ratio for the remaining loci was not significant. The details are given in Table 3.

Table 3. Chi square Segregation of all declared loci

Marker	Chi Squar 1:01	p	hmz	htz	n
CnCirB3	0.03	0.87278	20	19	39
CnCirC 11	0.03	0.85268	15	14	29
CNZ02	2.78	0.09558	23	13	36
CN11E10	2.94	0.08635	12	22	34
CNZ18	0.03	0.8618	16	17	33
CNZ01	1.13	0.28884	19	13	32
CNZA4	4.83	0.02799*	24	11	35
CNZ40	4.67	0.03075*	28	14	42

p - Probability of 1:1 segregation
hmz - Number of homozygotes (Probable)
htz - Number of heterozygotes (Probable)
n - Total

Table 1. Details of primers used in the analysis

Number	Primer	Forward primer (5`-3`) name	Reverse primer (3`-5`)
1	CnCirC11	TGTTATTTTGTTATTTCAGG	TCACCATCTTCTCAGTTTC
2	CNZ A4	CAGGATGGTTCAAGCCCTTAA	GGTGGAAGAGGGAGAGATTGA
3	CnCirB3	CATCTTGCTTTTCACCATCC	AATACTGTGCGGTTTTGCTT
4	CNZ 01	ATGATGATCTCTGGTTAGGCT	AAATGAGGGTTTGGAAGGATT
5	CN11 E10	TACTTAGGCAACGTTCCATTC	TAACCAGAAAGCAAAAAGATT
6	CNZ 40	CTTGATTGCTATCTCAAATGG	CTGAGACCAAATACCATGTGT
7	CNZ 18	TGATACTCCTCTGTGATGCTT	GTAGATTGTGGGAGAGGAATG
8	CNZ 02	CTCTTCCCATCATATACCAGC	ACTGGGGGGATCTTATCTCTG

Map of SSR loci

The distance between two genes is determined by their recombination frequencies. Kosambi map functions used were to convert the recombination frequency value into map distance. Total map size was 1086.4cM The map distance between CNZA4 and CNZ40 was the lowest (50.4CcM), followed by CNZA4 and CNZ01 (172.7cM), CNZ01 and CNZ18 (172.7cM), CNZ18 and CN11E10 (172.7cM), CN11E10 and CNZ02 (172.7 cM), CNZ02 and Cncir11 (172.7 cM), and between CncirC11 and CnCirB3 (172.2cM).

This preliminary study has reflected the map position of various microsatellite markers used and it did not show any linkage as the map distance between two SSR loci is greater than 5cM and the LOD score is lesser than 3.

The study shows the utility of the SSR markers in mapping studies of coconut. It demonstrates the utility of segregating progenies derived from selfing the heterozygous parent palm for mapping the linked loci. This study requires further analysis for getting more number of SSR loci to draw the linkage between various SSR loci.

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