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Research Papers

Analysis of population structure and genetic relatedness among root (wilt) disease-resistant and susceptible west coast tall coconut palms (*Cocos nucifera*) using microsatellite markers

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ABSTRACT

The population structures among the root (wilt) disease-resistant and susceptible coconut palms from 12 locations in the three disease-endemic districts of southern Kerala, were analyzed using nine microsatellite markers. The pair-wise population differentiation estimate (F_{st}) between the resistant and susceptible populations was 0.021. Two major populations and a subpopulation cluster were identified among the resistant palms. The analysis of genetic relatedness between the resistant mother palms showed that most of the palms located in a single locality shared sib relationship among them. The existence of close genetic relationship among resistant palms from Chengannur, Thiruvalla, Kottayam and Pavukkara localities in Kerala were reported. The results are discussed in terms of breeding strategies for increasing heterozygosity and obtaining the maximum number of disease-resistant seedlings.

Key words: Coconut, Genetic relatedness, Microsatellites, Resistance breeding, Root (wilt) disease

Coconut root (wilt) disease is an endemic disease in southern Kerala. Flaccidity, yellowing and necrosis of leaflets are reported to be the major distinguishing symptoms of the disease. According to a survey conducted in 1984, the disease was found to cause an annual loss of 968 million nuts (CPCRI 1985). In the contiguously diseased area of Kerala, vast majority of coconut palms have succumbed to the disease. Systematic investigations carried out indicated the association of phytoplasma (Solomon *et al.* 1983), transmitted by *Stephanitis typica* (lace bug) (Mathen *et al.* 1987) and *Proutista moesta* (plant hopper) (CPCRI 1991). As the disease could not be controlled by physical or chemical plant protection methods, the use of resistant or tolerant varieties has been regarded as the most ideal and practical method for the management of coconut plantations in the disease-prevalent tracts.

Since 1988 a comprehensive breeding programme for resistance/tolerance to coconut root (wilt) disease has been implemented at the Regional Station, Central Plantation Crops Research Institute (CPCRI), Kayangulam. The mother palms for the breeding programme were selected based on strict criteria (Nair *et al.* 1996) from the approximately one in a million coconut palms that appear to remain immune to the disease. Considering the better performance of the progenies produced from the field-resistant WCT palms, the Institute commenced establishing nucleus seed gardens by planting self- and sib-mated progenies of field-resistant WCT, for large-scale production of quality coconut seedlings (Nair *et al.* 2000). The seedlings produced from the first generation progenies are expected to have high yield and higher level of resistance to coconut root (wilt) disease.

These resistant palms occurring sporadically may be distinct genotypes evolved through natural selection/cross combinations. However, many of the selected mother palms succumb to root wilt disease after 10 –15 years of use in the resistance breeding programme, thus causing enormous wastage of land, labour and capital.

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The main objective of the present study is to develop a microsatellite database of the root (wilt)-resistant and susceptible palms. The identification and knowledge of distinct population clusters and genetic relationships among the resistant palms would help in breeding for maximizing the heterozygosity and therefore the disease resistance of the resultant progenies.

MATERIALS AND METHODS

Resistant west coast tall mother palms were selected from the ongoing resistance breeding progamme in the endemic disease areas (hotspots of disease) of Kottayam, Alappuzha and Pathanamthitta districts. Care was taken to locate susceptible palms in and around the resistant palms.

Unopened spindle leaf samples were collected from both the resistant and susceptible palms belonging to the same west coast tall cultivar in the sampled region.

In this study, palms were sampled at those locations in the Alapuzha, Pathanamthitta and Kottayam districts of Kerala, which had recorded the earliest incidence of the disease and formed the foci of the disease. Leaf samples were collected from 30 resistant mother palms in seven blocks, viz Chengannur (PT3 and PT6 located at Pandanadu; T776 and T779 located at Pavukkara), Kayangulam (PT4, PT8, PT10, PT11, PT13, PT19, PT40, PT42 located at Kayangulam) and Muthukulam (HT706, HT708, HT802, HT811, HT847 located at Haripad) in Alapuzha district, Thiruvalla (TT1, TT2 located in Manakachira) and Pulikheezhu (T789, T156, T178, T640 located at Valanajavattom, Mangat, Manipuzha and Theveri, respectively) in Pathanamthitta distict and Kottayam (T466, T414 and T415 located at Chingavanam, Kummavanam and Kiliroor, respectively), Vaikom (VL2, VL4 and VL5 located at Velloor; VT93 from Vaikom) in Kottayam district. All these mother palms are used in the root (wilt) resistance breeding programme to create selfed and sib-mated seedlings. Leaf samples were also collected for this study from a matching number of 30 susceptible palms (labelled as ST 1 to ST 35) adjacent to the resistant mother palms at each location.

DNA was extracted from the spear leaf as per the procedure of Upadhyay *et al*. 2000. Five gram of spear leaf tissue was ground in liquid nitrogen and transferred to extraction buffer containing 10% SDS. The contents were boiled at 65°C, cooled, and extracted with an equal volume of 24:1 chloroform: isoamyl alcohol mixture. The supernatant was transferred to a new tube and DNA was precipitated with 70% ethanol.

Microsatellite analysis was carried out with the following conditions. Each well received 12.5 ng of DNA, 200 µM dNTPS, 1U of Taq polymerase (Bangalore Genie, India), 1 µM of each primer. The PCR conditions were identical to that of Perera *et al*. 2000. The amplified products were resolved in a 5% denaturing polyacrylamide gel and the bands were visualized by silver staining (Bassam and Caetano-Anollés 1993). The microsatellite bands were scored manually and the alleles were sized with reference to a 30–330 bp ladder (Gibco Brl). The morphological observations and serological analysis was carried twice a year for six years till June 2009.

The calculation of genetic diversity values and construction of the UPGMA dendrogram using Nei's (1972) minimum genetic distance was carried out using the POWERSSR v 1.2 software (Liu 2001). The analysis of molecular variance (AMOVA) was done using GENALEX software (Peakall and Smouse 2001) with significance setting permutation value of 999. The Hardy -Weinberg equilibrium test was conducted using GENEPOP software.

The population structure in the resistant and susceptible palms was analyzed using the structure software. The burn-in duration and number of iterations for the MCMC was optimized by several trial runs with the data. A burn-in duration of 50 000 runs and 200 000 MCMC simulations were found to be optimal for this study. The posterior probability was calculated for sub-populations ranging from 1=10 using the default settings, admixture model and allele frequency correlated model from 20 replicated runs. The optimal K value was selected following instructions in the structure manual (Pritchard *et al.* 2000) and verified by the statistical method proposed by Evanno *et al.* 2005. The genetic relatedness of the individual resistance palms were studied using the ML-relate software (Kalinowski *et al.* 2006).

RESULTS AND DISCUSSION

Genetic diversity

A total of 49 alleles were detected by the nine microsatellite loci with an average of 5.4 alleles/locus. A significant difference in allele frequencies between the resistant and susceptible populations were noticed for the 151 bp allele of CnCIRE2, 199 bp allele of CnCIRF2 and 154 and 160 bp alleles of CAC6 and 187 bp allele of CAC3 primers. The heterozygosity or gene diversity between the resistant and susceptible populations was significantly altered for the CAC13 primer (0.621/0.238), while the rest of the loci did not show significant difference (Table 1). The primers CAC11 and CAC13 detected three alleles while the primer CnCIR E2 detected 11 alleles.

The within and between population variation between the resistance and susceptible population was 98% and 2%, respectively. A statistically significant deviation from the Hardy- Weinberg equilibrium was observed for the resistant population indicating an evolutionary selection pressure for specific resistant genotypes is in operation. The genotypic disequilibrium test conducted for each pair of loci indicated a non-significant LD in both the resistant and susceptible population. The level of genic differentiation between the resistant and susceptible reference populations was not significant. A pair-wise F_{st} estimate of 0.021 was observed

Table 1 Details of microsatellite loci, alleles detected and gene frequency and diversity in the resistant and root (wilt) disease-susceptible coconut populations

Microsatellite	Alleles (bp)	Gene frequency		Gene diversity	
		Resistant	Susceptible	Ho Res/Sus	He Res/Sus
CAC3	187	0.183	0.375		
	197	0.283	0.232		
	199	0.25	0.286	0.767/	0.767/
	201	0.233	0.107	0.500	0.712
	203	0.05	0		
CAC4	182	0.232	0.231		
	186	0.089	0.058	0.321/	0.673/
	188	0.179	0.154	0.423	0.629
	200	0.482	0.53		
	204	0.018	0.019		
CAC6	150	0.232	0.25		
	152	0.232	0.146	0.357/	0.808/
	154	0.125	0.042	0.375	0.802
	156	0.232	0.22		
	158	0.054	0.083		
	160	0.107	0.229		
	162	0.018	0.021		
CAC8	188	0.15	0.107		
	196	0.117	0.125	0.633/	0.684/
	198	0	0.018	0.429	0.719
	200	0.083	0.089		
	202	0.15	0.214		
	204	0.5	0.446	0.458/	0.513/
CAC10	195	0.083	0.146	0.583	0.577
	197	0.646	0.563		
	201	0.021	0.292	0.286/	0.436/
	203	0.25	0	0.292	0.405
CAC11	156	0.321	0.25	0.621/	0.615/
	158	0.679	0.729	0.238	0.414
	170	0	0.021		
CAC13	158	0.259	0.071		
	162	0.224	0.19	0.345/	0.300/
	172	0.517	0.738	0.321	0.480
CnCIRF2	193	0.828	0.679		
	195	0.017	0		
	197	0.017	0.071		
	199	0.121	0.232		
	205	0.017	0.018		
CnCIRE2	115	0.077	0.08	0.462/	0.814/
	129	0.058	0.06	0.520	0.734
	137	0.019	0.02		
	151	0.269	0.46		
	153	0.038	0.02		
	155	0	0.06		
	157	0.058	0.04		
	163	0.269	0.18		
	171	0.058	0		
	173	0.154	0		
	175	0	0.08		

between the resistant and susceptible population. The pair-wise population genetic identity of 0.933 was observed between the resistant and susceptible population.

Private alleles: Five and four private alleles were observed in the resistant and susceptible samples, respectively. The primer CnCIRE2 showed a maximum of two private alleles each in the resistant and susceptible samples (Table 2).

UPGMA dendrogram

Overall two groupings and two sub-clusters in each of these two groping could be detected among the resistant and susceptible palms in the UPGMA dendrogram constructed with Nei's standard genetic distance (1972) (Fig 1).

The first grouping consists mainly of the resistant palms from the Kayangulam, Haripad, Vaikom Velloor and Valanjavattom. The second grouping consist palms mainly from the Thiruvalla, Kottayam, Pavukkara, Mangat and Theveri.

The resistant talls from Kayangulam (six of them, PT19, PT10, PT42, PT13, PT40, PT8), Haripad (two of them, HT811 and HT802), Vaikom (one palm VT 93) and Velloor (one palm, VL 5) formed a sub-cluster in the first grouping. The resistant talls from Thiruvalla (TT1 and TT2), Kottayam (T414, T415 and-416) and Mangat (T156) formed a sub-cluster in the second grouping. The single resistant palms PT8 from Kayangulam and T640 from Theveri formed a single sub-cluster in the second groupings. Similiarly, three groups of susceptible palms formed sub-cluster with the resistant Tall groups.

Population structure analysis

The structure software identified four and three population clusters among the resistant and susceptible palm populations together, respectively, following the procedures of Pritchard *et al.* 2000 and Evanno *et al.* 2005 (Table 3). Among the four clusters identified three clusters had palms from both resistant and susceptible populations, while the fourth cluster comprised mainly the resistant palms from Kayangulam, Haripad, Vaikom, Pavukkara and Valanjavattom. Among the resistant mother palms, three populations were identified following the procedures of Pritchard *et al.* 2000 while two populations and a subpopulation were identified with the procedures of Evanno *et al.* 2005. Four populations were identified in the susceptible palms by both these statistical procedures.

Genetic relatedness of resistant mother palms

The genetic relationship studies showed that not all the resistant mother palms located nearby shared a close relationship. The two resistant palms from Chengannur (PT3 and 'PT6') and Thiruvalla (TT1 and TT2) were identified as full siblings. The resistant palm PT4 from Kayangulam was unrelated to all other resistant palms except for the T178 palm from Manipuzha to which it shares a close (Parent-

Table 2 Private alleles observed in the coconut (root) wilt resistance and susceptible populations

Population	Locus	Allele (bp)	Freq
RES	CAC10	203	0.250
RES	CAC3	203	0.050
RES	CnCIRF2	195	0.017
RES	CnCIRE12	171	0.058
RES	CnCIRE12	173	0.154
SUS	CAC8	202	0.018
SUS	CAC11	170	0.021
SUS	CnCIRE12	155	0.060
SUS	CnCIRE12	175	0.080

offspring) relationship. The resistant mother palm VL 5 located at Velloor shares the PO relationship with the maximum number of four of the resistant palms studied (PT19, VT93, HT802, HT811) (Table 4). The resistant mother palm HT786 was unrelated to all other palms except for the 'PT11' for which it has a full sib relationship.

All the palms are about the same age and hence cannot share a parent-offspring relationship. Despite this limitation, in the absence of the parental palms of these resistant palms, the genetic relatedness study gives an estimate of the degree of relationship between individual resistant mother palms. This information can be used in the parental selection for exploiting the heterosis and disease resistance among the resistant mother palms.

The identification of two-population structure among the resistant mother palms will help to reorient and fine-tune the breeding programme. The progenies resulting from the cross between these two diverse resistant populations are expected to have high heterozygosity and hence enhanced disease resistance.

The disease affects coconut seedlings even at the nursery stage. Nearly 30–60% of the progenies from crosses between the resistant mother palms become diseased. A majority of the palms that are diseased before the bearing age either produce no inflorescence at all or very small inflorescences that are dried out on eventual emergence. Disease escape may not be a suitable explanation for the apparently healthy palms as strict criteria (e g surrounded by more than 80% diseased palms) are applied before their selection and utilization in the breeding programme. Moreover, the disease is endemic to these districts for more than 100 years and, as the economic bearing age of individual coconut palm ranges from 35 to 60 years, at least 2–3 generations of palms might have responded to selection pressure by evolving resistant genotypes. The fact that the resistant mother palms remain healthy for more than 50 years and yield well during their economic productivity stage is an indication of their potential application in the breeding programme. The breakdown of resistance in some mother palms after 50 years may be due to aging or emergence of new virulence factors and

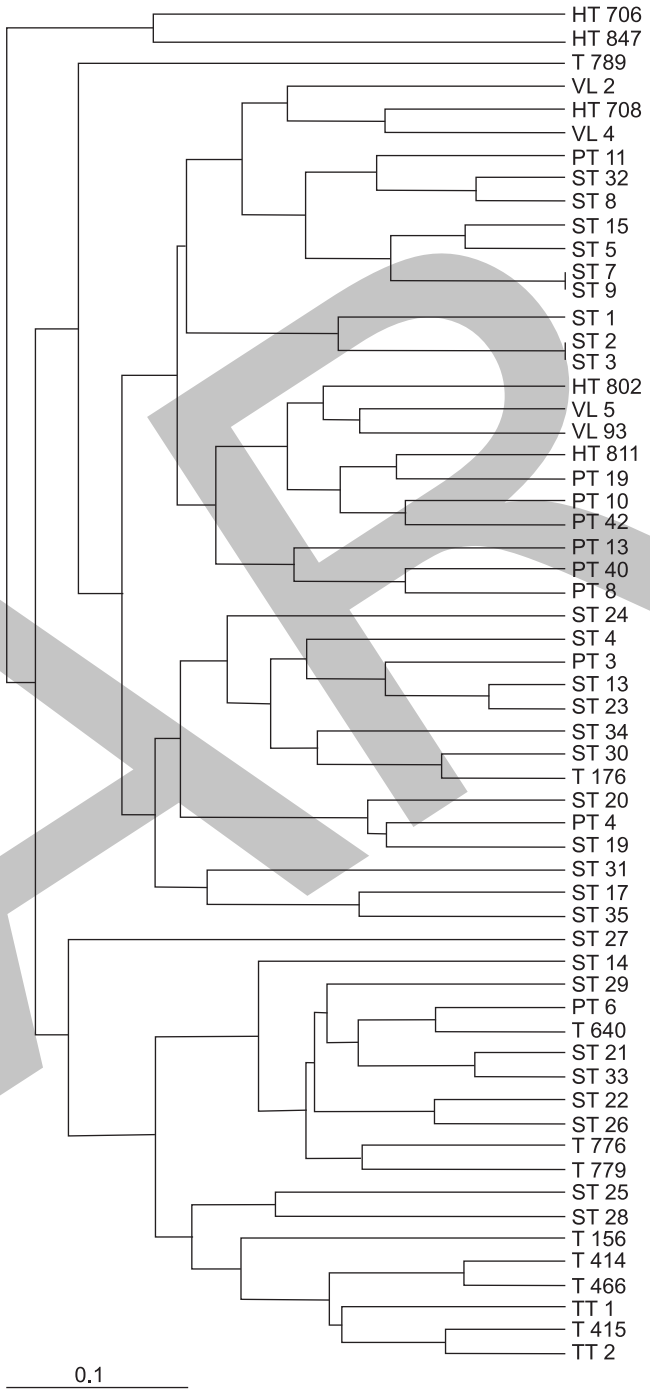

Fig 1 UPGMA Dendrogram depicting the relationship between the individual root (wilt)-resistant and susceptible genotypes (Nei's standard genetic distance 1972)

breakdown of resistance has been reported in the case of coconut lethal yellowing disease. The construction of linkage maps of susceptible and resistant palms is not possible because the susceptible palms yield very few nuts while the reciprocal crosses are time consuming given the palm's long (4–6 year) juvenile phase and the long period (at least 35

years) required for the phenotypic observation on resistance/ susceptibility. Identifying microsatellite genotypes that are possibly linked to the resistance trait along with morphological observations is the practical way to breed for root (wilt) disease resistance.

Since the collection of leaf samples for analysis, it was observed that six palms (VL 4, VL 5 from Velloor, RT5 from Chengannur and PT 4, PT 8 and PT 42 from Kayangulam) were found to be serologically and morphologically diseased. Despite this setback, the resistance breeding programme is being continued with the identification of more number of resistant parental palms in the endemic tract and utilizing them in the breeding programme.

The present root (wilt) disease resistance breeding strategy aims at increasing the homozygosity by selfing and sib-mating between palms in a single locality. This has resulted in inbreeding depression in the form of very low percentage of nut setting and disease susceptibility of the resultant progenies from these crosses (data not shown). Our study may help resistance breeders to reorient their breeding strategies by making rational choice of parental combinations with divergent genotypes thus increasing the better performance of the progenies.

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Table 3 Population clusters in root (wilt)-resistant and susceptible palms as identified by structure (v.2.0) software

Population	No of clusters identified		F_{st} variance* among the clusters (±SD)				Palm grouping			
	(Pritchard *et al.* 2000)	(Evanno *et al.* 2005)					I	II	III	IV
Resistant and susceptible population (60 palms)	4	3	0.203 (±0.04)	0.226 (±0.05)	0.225 (±0.05)	0.210 (±0.04)	RES VL 2, VL 4, VL 5, HT 708 SUS ST 2, ST 3, ST 5, ST 32, ST 12, ST 31, ST 7, ST 8	RES T414, T466, T415, TT2, TT1, T156, T640, PT6, T776, T 779 SUS ST 22, ST 26, ST 33, ST 21, ST 9, ST 25, ST 29, ST 28, ST 14, ST 35	RES PT 4, T178, PT 3 SUS ST 20, ST 23, ST 30, ST 34, ST 10, ST 24, ST 4, ST 27, ST 17, ST 1	RES PT 13, PT 40, PT 10, HT847, T789, PT 42, T779, HT706, HT 811, PT 11, VT 93, PT 8, HT 802
Resistant population (30 palms)	3	2(1)	0.131 (±0.07)	0.191 (±0.11)	0.219 (±0.10)		PT3, PT6, TT1, TT2, T466, T 414, T 415, T 156, T 640, T 776, T779	PT10, PT13, PT19, PT40, PT42, VT93, HT802, HT811, HT847, VL5	HT706, PT4, PT11, HT708, T178, PT8, VL2, VL4, T789 ST 20, ST 19, ST 31, ST 13, ST 27	
Susceptible population (30 palms)	4	4	0.255 (±0.13)	0.313 (±0.09)	0.273 (±0.13)	0.268 (±0.08)	ST 6, ST 7, ST 5, ST 32, ST 1, ST 2, ST 3, ST 15	ST 26, ST 22, ST 33, ST 21, ST 9, ST 14, ST 29, ST 28		ST 34, ST 30, ST 23, ST 17, ST 24, ST 25, ST 4, ST 35

Table 4 Genetic relationship of the individual resistant palms as inferred by the nine microsatellite primers used in this study

	PT3	PT6	TT1	TT2	T466	T414	T415	T156	T178	T640	T776	T779	T789	PT4	PT8	PT10	PT11	PT13	PT19	PT40	PT42	VT93	HT706	HT708	HT802	HT811	HT847	VL2	VL4	VL5
PT3	-																													
PT6	FS	-																												
TT1	FS	HS	-																											
TT2	U	U	FS	-																										
T466	U	HS	PO	FS	-																									
T414	U	U	HS	FS	FS	-																								
T415	U	HS	FS	FS	HS	PO	-																							
T156	U	HS	HS	FS	HS	PO	FS	-																						
T178	U	U	U	U	U	U	U	U	-																					
T640	U	FS	PO	U	U	U	HS	U	U	-																				
T776	HS	HS	PO	HS	HS	U	FS	HS	HS	PO	-																			
T779	U	HS	FS	HS	HS	HS	PO	PO	PO	U	U	FS	-																	
T789	U	U	U	U	HS	HS	U	HS	PO	U	U	U	-																	
PT4	U	U	U	U	U	U	U	U	PO	U	U	U	U	-																
PT8	U	U	U	U	U	U	U	U	U	U	U	U	PO	PO	-															
PT10	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	-														
PT11	U	U	U	U	U	U	U	U	U	U	U	HS	FS	U	U	U	-													
PT13	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	HS	HS	FS	-												
PT19	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	-											
PT40	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	FS	U	U	FS	HS	-										
PT42	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	PO	FS	U	U	FS	U	-									
VT93	U	U	U	U	U	U	U	U	U	U	U	U	HS	FS	FS	U	HS	FS	U	FS	-									
HT706	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	U	U	U	U	U	U	-							
HT708	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	U	U	U	U	U	HS	U	-						
HT802	PO	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	HS	U	PO	U	U	-					
HT811	PO	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	HS	U	PO	U	U	-				
HT847	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	U	HS	HS	U	U	HS	U	HS	HS	-			
VL2	U	U	U	U	U	U	U	U	PO	U	U	U	U	U	U	U	U	U	HS	U	U	HS	U	U	U	U	U	-		
VL4	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	U	U	U	U	FS	U	FS	U	HS	U	FS	-	
VL5	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	U	U	PO	U	U	PO	PO	U	U	PO	-

U Unrelated; FS, full sib; HS, half sib; PO, parent offspring.

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