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Genetic Variability of Corynespora cassiicola *Populations in Thailand*

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Differential clones and random amplified polymorphic DNA markers were used to investigate the genetic diversity among twenty-four isolates of Corynespora cassiicola *from rubber plantations in Thailand. According to the virulence reaction, the isolates were classified into eleven pathotypes. However, cluster analysis of RAPD data separated isolates into three groups and no correlation between different RAPD groups, pathotype or geographical region was detected. Analysis of the molecular variance revealed that 54.97% of total genetic diversity was due to variation among isolates within the regions and 45.03% came from variation between regions. The genetic differences in* C. cassiicola *populations provide important information for studies on disease resistance which will be useful in the choice of breeding strategies to develop resistant rubber clones.*

Key words: *Corynespora cassiicola*; *Hevea brasiliensis*; RAPD; pathotype; genetic variability; DNA markers; disease resistance; breeding; Thailand

Corynespora leaf disease, caused by *Corynespora cassiicola* (Berk. & Curt.) Wei, is considered as one of the major diseases in rubber plantations worldwide. In 1958, the disease was first reported in a seedling nursery in India[1]. Later, several reports on the disease were published in many countries[2–5]. The pathogen can infect rubber trees at all growth stages. The disease symptoms can be visualized as black spots on leaves or black lesions on petioles and green twigs. Subsequently leaf fall occurs. The unique characteristic of this disease is the fish-bone or railway track-like appearance of dark brown lesions on leaves. Disease severity depends on the level of resistance to the disease. In susceptible immature rubber, the pathogen causes repeated leaf fall all year round. Hence, the tree has less leaves, is retarded in growth, and finally dies. In susceptible mature rubber trees, the disease significantly decreases latex production. Approximately 20%–25% economic losses in

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rubber production by this disease have been reported[6]. In Thailand, severe infection of this pathogen causing crop loss was first reported in 1985 on rubber clones[4] RRIC 103 and RRIT 21. At present, the pathogen can be found in all rubber plantations in Thailand. Even the most widely grown RRIM 600 which is classified as moderately resistant, has become highly susceptible[7,8]. These observations suggest that the pathogen can adapt easily to rubber clones, and it is suspected that this will cause disease outbreak in the future. Therefore, the genetic information of the pathogen would be useful for better understanding of disease outbreaks, predicting future disease development, and developing effective strategies in breeding for disease resistance[9,10]. The information on population genetics of this disease will also reveal the evolutionary history of the pathogen[11]. Although conventional methods such as cultural morphology, growth characteristics, spore morphology and host specificity have been used for identification of genetic variation in pathogen populations[3,5,12,13], these assays are subject to environment variation. In recent years, several DNA markers technologies have been developed and used in genetic studies of many organisms[14,15]. However, since RAPD markers technology had been successfully used in a previous study on genetic variability among *C. cassiicola* isolates in Australia and Sri Lanka[16], the RAPD technique was selected for the present study on the pathogen isolated from rubber trees in Thailand.

MATERIALS AND METHODS

Isolates of *Corynespora cassiicola*

Twenty-four isolates of *C. cassiicola* studied were obtained from the Plant Pathology Laboratory, Rubber Research Institute of Thailand. The pathogen populations were collected from three different geographic locations in Thailand. The populations comprised six, four and fourteen isolates from the northeastern, eastern and southern regions, respectively (*Table 1*, *Figure 1*).

Pathogenic Variation

The pathotype of *C. cassiicola* isolates were identified based on characterisation of disease reaction on 13 differential clones of *Hevea*: AVROS 2037; BPM 24; GT 1; Haiken-1; Haiken-2; PB 255; RRIC 100; RRIC 101; RRIC 110; RRIM 600; RRIT 21; RRIT 250; and Tjir 1. Inoculation was performed by positioning a 6 mm diameter mycelial disc on the abaxial side of uniform, fully expanded leaflets and incubated in moist chambers at room temperature. Disease severity was scored by observation of reaction symptoms three days after inoculation. A score 0, 1, 2 or 3 was used to indicate no visible symptom, small dark discolouration below mycelial disc, prominent large lesion without extended blackish veins or prominent large lesion with extended blackish veins, respectively. An average disease severity for each host/isolate combination was calculated and values of <1, ≥ 1 and ≥ 2 were used to classify reaction as resistant (R), moderately resistant (MR) and susceptible (S), respectively. Each host/isolate combination was tested in three replications with four leaves per replicate.

DNA Isolation

Mycelia were grown in 50 mL of potato dextrose broth in 200 mL Erlenmeyer flasks and incubated at room temperature for 7 days. After filtration and washing with sterile distilled water, 0.4 g of frozen mycelium was ground in a mortar with liquid nitrogen. DNA

TABLE 1. SOURCE OF *CORYNESPORA CASSIICOLA* ISOLATES USED IN THE STUDY

No.	Isolate	Location	Geographical origin
1	NE-01	Muang, Loei	Northeast
2	NE-02	Muang, Loei	Northeast
3	NE-03	Phu Phan, Sakon Nakhon	Northeast
4	NE-04	Sahatsakhan, Kalasin	Northeast
5	NE-05	Det Udom, Ubon Ratchathani	Northeast
6	NE-06	Muang, Nakhon Phanom	Northeast
7	E-01	Phanom Sarakham, Chachoengsao	East
8	E-02	Sanam Chai Khet, Chachoengsao	East
9	E-03	Phanat Nikhom, Chon Buri	East
10	E-04	Ban Khai, Rayong	East
11	S-01	Tha Chana, Surat Thani	South
12	S-02	Muang, Surat Thani	South
13	S-03	Muang, Yala	South
14	S-04	Palian, Trang	South
15	S-05	Muang, Trang	South
16	S-06	Thai Muang, Phangnga	South
17	S-07	Hat Yai, Songkhla	South
18	S-08	Chawang, Nakhon Si Thammarat	South
19	S-09	Than To, Yala	South
20	S-10	Khok Pho, Pattani	South
21	S-11	Nabon, Nakhon Si Thammarat	South
22	S-12	Takua Pa, Phangnga	South
23	S-13	Ruesoe, Narathiwat	South
24	S-14	Muang, Krabi	South

extraction was performed by the method described by Roger and Bendich[17]. DNA concentration was estimated by comparing band intensity with known quantities of phage lambda DNA in agarose gel electrophoresis. The final DNA concentration was adjusted to approximately 5 ng/µL and stored at –20ºC.

RAPD Analysis

Based on stable amplification, 15 arbitrary primers from Operon Technologies (OPC-01, OPC-05, OPC-06, OPC-11, OPC-16, OPD-02, OPD-03, OPD-13, OPD-14, OPD-15, OPD-16, OPL-12, OPM-03, OPM-13 and OPN-14) were

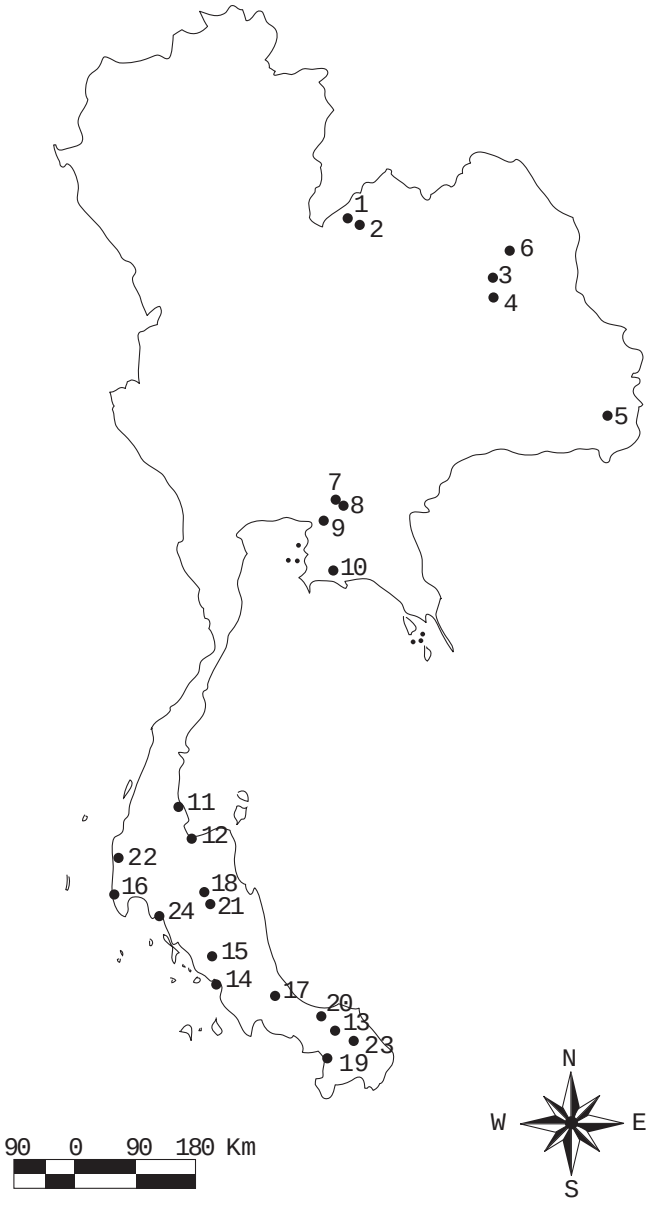

Figure 1. Map of Thailand showing the location of rubber plantations from which the Corynespora cassiicola *were collected. Numbers indicate the populations of the fungus listed in* Table 1.

selected for this experiment. The PCR reactions were carried out in a final volume of 50 µL containing 1X DNA polymerase buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 0.21 mM each of dATP, dCTP, dGTP and dTTP, 0.6 µM primer, 1 unit of Taq DNA polymerase, and 5 ng of genomic DNA. Amplifications were performed in a thermal cycler with initial step at 94ºC for 3 min, followed by 35 cycles at 94ºC for 45 s, 35ºC for 1 min, and 72ºC for 2 min, and a final cycle at 72ºC for 10 min. The amplification products were resolved in 2.0% agarose gel (UltraPure Agarose, GIBCO BRL) under 0.5X TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 60 V for 1.5 h at room temperature. Gels were stained with ethidium bromide, and visualized with a UV transilluminator. The sizes of amplified DNA fragments were estimated by comparison with a 1 Kb Plus DNA Ladder marker (Invitrogen Corp., CA). To confirm consistent amplification, all PCR reactions were performed in three replications.

Data Analysis

The stable amplified DNA fragments were scored as 1 for presence and 0 for absence of amplified fragment. The binary matrix data were then used for analysis of Nei and Li similarity index[18] and for genetic differentiation among the pathogen populations. Cluster analyses were performed from the genetic distance matrices by the unweighted pair-group method with arithmetic mean averages (UPGMA) using 1000 bootstrapped replications, with PHYLIP package software version 3.6 (alpha 3)[19]. Phenograms were displayed using TREEVIEW software[20].

Analysis of molecular variance (AMOVA)[21] was carried out to hierarchically partition genetic diversity among isolates within and between populations. The AMOVA was performed by ARLEQUIN 2.0 software[22] based on a distance matrix computed by pairwise difference method, and significance test using 1000 permutations.

RESULTS AND DISCUSSION

Pathogenic Variability

According to the disease reactions from differential screening, the 24 isolates of *C. cassiicola* were classified into 11 pathotype groups, A to K (*Table 2*). Group A (avirulence) and group K (virulence) were the most common, comprising, respectively, 20.83% and 41.67% of the total samples. Pathogenic variability among *C. cassiicola* isolates had been described in many countries but the large difference in *Hevea* differential clones employed make it difficult to compare the results. However, there was no obvious pattern linking virulence with host genotype or geographical origin in this study.

Genetic Differentiation among *C. cassiicola*

A total of 152 consistently amplified DNA bands were generated from fifteen RAPD primers. The size of DNA fragments ranged from 250 bp to 4500 bp and the examples of the DNA pattern from RAPD are shown in *Figure 2*. Ninety-eight percent of RAPD bands were polymorphic among the 24 isolates. However, two isolates, S05 and S08, showed identical RAPD phenotype. The polymorphism levels within sub-populations were 25.66% for the northeast, 61.18% for the south, and 85.53% for the east. Five RAPD primers gave no polymorphism in the northeastern sub-population (*Table 3*). The results also

TABLE 2. REACTION OF *HEVEA* CLONES TO *CORYNESPORA CASSIICOLA* ISOLATES

Hevea clone											Pathotype group	Isolates
AVROS 2037	BPM 24	GT 1	Haiken-1	Haiken-2	PB 255	RRIC 100	RRIC 101	RRIC 110	RRIM 600	RRIT 21	RRIT 250	Tjir 1		
R	R	R	R	R	R	R	R	R	R	R	R	R	A	NE04, E01, E03, S09, S12
R	R	R	R	MR	MR	R	R	R	R	R	R	R	B	S13
R	MR	R	R	MR	R	MR	MR	R	R	R	MR	R	C	NE02
R	R	R	R	MR	R	MR	MR	R	S	R	S	R	D	S02
R	R	MR	MR	S	MR	S	R	MR	S	R	MR	R	E	S01
R	MR	S	MR	MR	R	S	R	S	S	R	MR	S	F	NE01
MR	S	MR	R	R	R	S	R	S	S	S	S	MR	G	NE05
R	S	S	R	MR	MR	S	S	S	R	S	MR	MR	H	S11
R	S	S	R	MR	S	S	S	S	S	S	MR	S	I	S10
R	S	S	S	MR	S	S	S	S	S	S	MR	S	J	NE03
S	S	S	S	S	S	S	S	S	S	S	S	S	K	NE06, E02, E04, S03, S04, S05, S06, S07, S08, S14

R = Resistant; MR = Moderately resistant, S = Susceptible

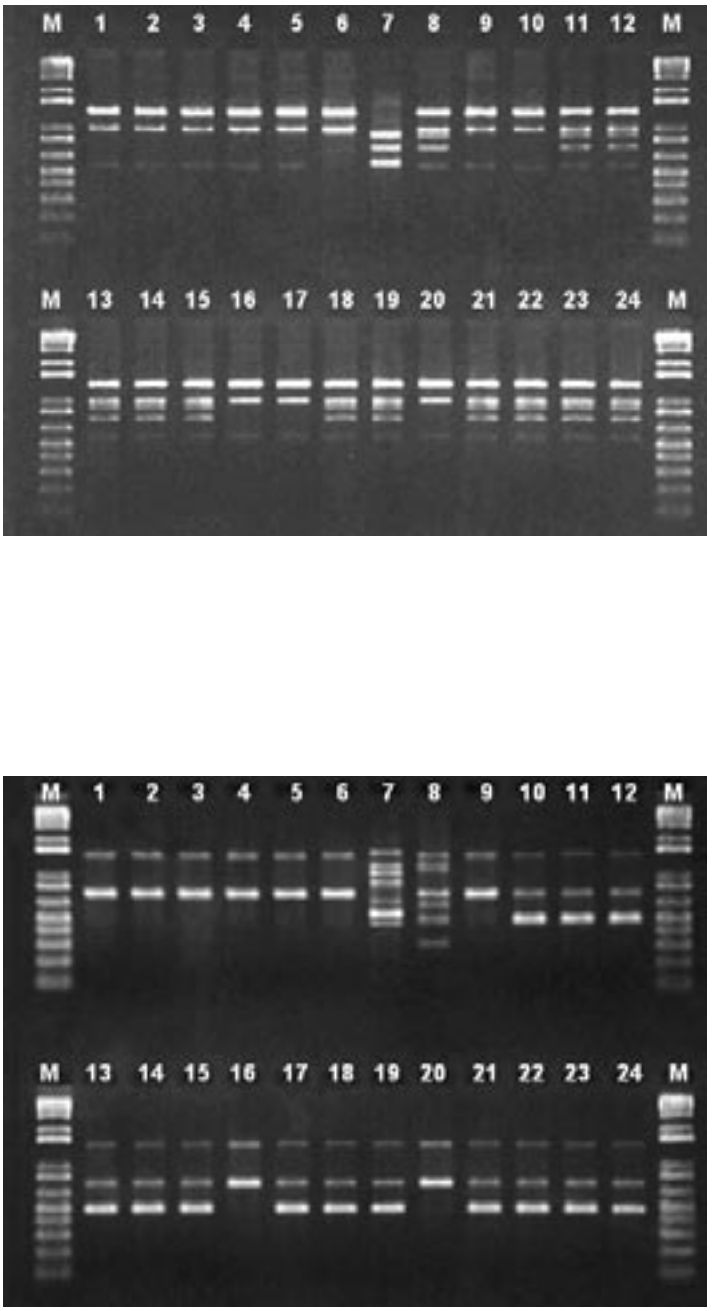

Figure 2. Random amplified polymorphic DNA profiles of Corynespora cassiicola *isolates generated with primer OPD-02 (upper) and primer OPM-03 (lower). Lanes 1 to 6 represent the northeastern isolates; lanes 7 to 10 represent the eastern isolates; and lanes 11 to 24 are the southern isolates. M corresponds to 1 Kb Plus DNA Ladder (Invitrogen Corp., CA) molecular size marker.*

indicated that RAPD analysis is a potential technique for discrimination of *C. cassiicola* isolated from rubber as shown by Silva *et al.*[16]

The lowest genetic variation in the northeastern sub-population was expected since the rubber plantation had been introduced in this area not much later compared to the southern and eastern regions. The traditional rubber planting area originated in the south and extended to the east and then to the northeast in the recent years. Considering the period of rubber plantation, the highest genetic diversity of the pathogen population should be found in the south. However, the genetic variation of the disease in the south was lower than in the east. This was probably due to insufficient DNA markers used in this study or the data generated from RAPD primers cannot differentiate between the heterozygous from the homozygous effects. Thus, the variation causes by cross-breed among the pathogens were not detected. Differences in climate between the south and the east may also play an important role for genetic diversity of the disease as described by Leung *et al.*[9]

Population Structure

Data from cluster analysis using UPGMA are shown in *Figure 3*. At approximately 60% similarity, the pathogens were classified into 3 separate groups. Group I contained only isolate E01 collected from the east. Group II contained mainly isolates from the south with 2 isolates, E02 and E04, from the east. All isolates from the northeast were clustered in the

TABLE 3. NUCLEOTIDE SEQUENCE OF PRIMERS AND PERCENTAGE OF POLYMORPHIC LOCI FOR RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) LOCUS IN *CORYNESPORA CASSIICOLA* POPULATIONS FROM THREE REGIONS OF THAILAND

RAPD locus	Nucleotide sequence (5'→3')	Polymorphic loci (%)		
		Northeast	East	South
OPC-01	TTCGAGCCAG	41.67	91.67	66.67
OPC-05	GATGACCGCC	66.67	100.00	88.89
OPC-06	GAACGGACTC	36.36	63.64	81.82
OPC-11	AAAGCTGCGG	42.86	92.86	64.29
OPC-16	CACACTCCAG	60.00	70.00	40.00
OPD-02	GGACCCAACC	0.00	100.00	40.00
OPD-03	GTCGCCGTCA	14.29	78.57	71.43
OPD-13	GGGGTGACGA	8.33	91.67	50.00
OPD-14	CTTCCCCAAG	0.00	100.00	75.00
OPD-15	CATCCGTGCT	26.67	66.67	60.00
OPD-16	AGGGCGTAAG	9.09	81.82	81.82
OPL-12	GGGCGGTACT	0.00	83.33	50.00
OPM-03	GGGGGATGAG	0.00	100.00	9.09
OPM-13	GGTGGTCAAG	66.67	83.33	50.00
OPN-14	TCGTGCGGGT	0.00	100.00	75.00
Average		25.66	85.53	61.18

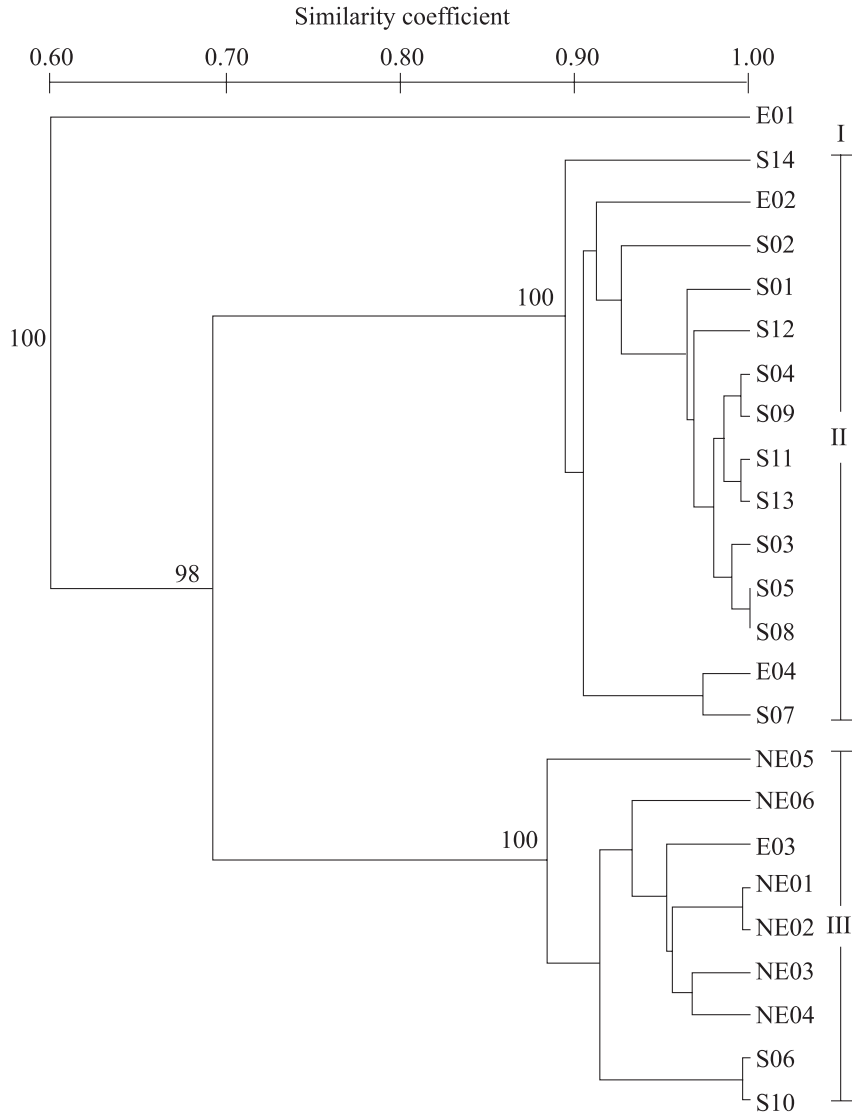

Figure 3. Phenogram of Corynespora cassiicola *populations collected from rubber plantations in Thailand based on random amplified polymorphic DNA, using unweighted pair-group method with arithmetic average. [Branch length is proportional to the genetic distance among isolates; bootstrap values of major clusters (based on 1000 replications) are indicated on internal branches; source of isolates are as given in* Table 1*].*

last group with an addition of 2 isolates, S06 and S10, from the south and an isolate E03 from the east. Considering the history of rubber plantations in Thailand, the rubber clones planted in the northeast and the east originated from the south. Thus, it is not surprising that the clusters in the phenogram did not show any relationship between different group and their geographic region. The bootstraped values for the clusters of nearly 100%, confirm the reliability of the differences.

The pair-wise comparisons of *C. cassiicola* populations showed strong differentiation between the northeastern and the southern sub-populations with high significance G_{ST} values (G_{ST} = 0.632, P = 0.000) while the genetic diversity between the northeastern and the eastern sub-populations was low (G_{ST} = 0.341, $P < 0.001$). No differentiation was found between the eastern and the southern sub-populations (G_{ST} = 0.124) (*Table 4*).

From the AMOVA, 54.97% of total genetic variation was attributed to variation among isolates within sub-populations and 45.03% for variation among sub-populations (*Table 5*). This analysis also showed a high significant difference among sub-populations (ϕ_{STAT} = 0.45, $P < 0.001$). Unfortunately, there is no

TABLE 4. PAIR-WISE COMPARISONS OF NEI'S COEFFICIENT OF GENETIC DIFFERENTIATION (G_{ST}) VALUES AMONG *CORYNESPORA CASSIICOLA* POPULATIONS FROM THREE RUBBER PLANTING REGIONS OF THAILAND DETERMINED BY RANDOM AMPLIFIED POLYMORPHIC DNA MARKER[a]

Populations	Northeast	East	South
Northeast	0.000		
East	0.341**	0.000	
South	0.632**	0.124	0.000

[a]Genetic diversity between populations as proportion of total diversity: $G_{ST} = (H_T - H_s)/ H_T$ where H_T = total genetic diversity within the species and H_s = genetic diversity for each population. Null hypothesis of no population differentiation. Asterisks indicate G_{ST} values are significant at $P < 0.001$.

TABLE 5. ANALYSIS OF MOLECULAR VARIANCE FOR THREE *CORYNESPORA CASSIICOLA* SUB-POPULATIONS ISOLATED FROM RUBBER TREES GROWING IN THAILAND, USING RANDOM AMPLIFIED POLYMORPHIC DNA PHENOTYPES

Source of variation	df	Variance	% Total variance	ϕ_{STAT}	P [a]
Among sub-populations (regions)	2	12.117	45.03	0.450	<0.001
Among isolates within sub-populations (regions)	21	14.793	54.97	–	–

[a]Probability of having an equal or more extreme variance component and ϕ statistic than the observed values by chance alone. (Tested by non-parametric randomisation analysis, using 1000 repetitions).

report concerning population structure of *C. cassiicola* available for comparison.

Molecular markers can provide a good assessment of the population structure; however, they do not tell us anything about pathotypic variation. There was no correlation between pathotype and RAPD haplotype, indicating a lack of association between genetic polymorphism and virulence.

Considering the pathogenicity and the high genetic diversity of *C. cassiicola*, it is clear that *Corynespora* leaf disease resistance should be screened with more isolates and locations. It would require a more extensive survey of the pathogen populations from a wide range of host plants for a better understanding of the origin of genetic variation and a greater knowledge of the resistant gene present in rubber clones. The research presented here provides the experimental basis for further investigations.

CONCLUSION

Analysis of *C. cassiicola* from rubber in Thailand using RAPD markers demonstrated that there was no correlation between RAPD haplotype and virulence phenotype or geographic region. The total genetic variation came from variation among isolates within sub-populations (54.97%) and from variation among sub-populations (45.03%). The knowledge of isolates and locations should be an important consideration in disease screening for genetic structure.

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