J. Rubb. Res., **17(3)**, 187–203

Genetic Diversity and Pathogenicity of Corynespora cassiicola *Isolates from Rubber Trees and Other Hosts in Vietnam*

NGUYEN DON HIEU*, NGUYEN ANH NGHIA*#, VU THI QUYNH CHI* AND PHAN THANH DUNG*

A total of 38 isolates of Corynespora cassiicola *collected from rubber trees and other hosts including papaya* (Papaya carica)*, Ecdysanthera rosea, wild mango* (Irvingia malayana)*, white pumpkin* (Benincasa cerifera) *and sesame* (Sesamum indicum) *from different regions of Vietnam were studied for genetic diversity analysis using ribosomal DNA internal transcribed spacer (rDNA ITS) sequencing and inter simple sequence repeat (ISSR) markers. A single nucleotide polymorphism at the 135th base (Cytosine/Thymine) of the rDNA-ITS regions differentiated 38 studied isolates into two groups. Meanwhile, 88 DNA bands amplified by eight ISSR primers also generated a phylogenetic tree which clustered the studied isolates into two main groups. This was harmonious from that differentiated using the sequence of rDNA-ITS. Four of these studied isolates representing two genetic clusters and hosts were subjected to pathogenicity test using detached leaf assay and artificial inoculation in glasshouse on plantlets of seven rubber clones, white pumpkin and papaya. These isolates showed different virulence on these hosts, which was displayed by the variation in the percentage disease intensity (PDI) and average infection score (AIS). Though belonging to the same genetic cluster,* C. cassiicola *isolated from papaya was avirulent to white pumpkin, whereas the isolate from white pumpkin was virulent to papaya. Isolates from white pumpkin and papaya were virulent to all tested rubber clones. It seemed that the genetic relationship among isolates depends on the geographical region rather than the host source.*

Keywords: *C. cassiicola*; inter simple sequence repeat (ISSR); rDNA ITS sequence; single nucleotide polymorphisms (SNPs); pathogenicity test

Corynespora cassiicola is a cosmopolitan fungus which has been recorded in over 80 countries worldwide and more than 350 plant species[1]. On rubber trees (*Hevea brasiliensis*), the earliest detailed report on the occurrence of the disease caused by *C. cassiicola* was from India in 1958, followed by Malaysia, Nigeria, Indonesia, Brazil, Sri Lanka, Cameroon, Thailand, Bangladesh, Vietnam, Ivory Coast, Gabon and China[2–12]. Since the 1980's, the

*Rubber Research Institute of Vietnam, 236bis Nam Ky Khoi Nghia St., Ward 6, Dist. 3, Ho Chi Minh City, Vietnam.
Corresponding author (e-mail: anhnghia@gmail.com)

severity of Corynespora leaf fall disease has been on the increasing trend. The disease reached an epidemic scale, affected huge areas of rubber plantations in most rubber growing countries and caused severe economic losses[13].

Phylogenesis of this fungus have been widely studied using random amplified polymorphic DNA (RAPD) markers[14–19], PCR-RFLP[15,17,20], ISSR[21], sequence analysis of ribosomal DNA internal transcribed spacer (ITS) region[22–23], *acc5*, *ag4* and actin coding loci[23]. Some studies found correlation between the genetic groups and the features of the isolates *e.g*. geographical origin, host plant genotype, pathogenicity and growth of isolates[15,17,20,21–23] but other studies found no correlation[18,19]. This paper reported the genetic diversity of 38 *C. cassiicola* isolates obtained from rubber trees and some other hosts from different regions of Vietnam and the pathogenicity of four isolates representing the genetic groups and hosts of the studied isolates on seven rubber clones and two other hosts including papaya and white pumpkin.

MATERIALS AND METHODS

C. cassiicola Isolates

A total of 38 *C. cassiicola* isolates were collected from several rubber clones and other hosts in different geographical locations in Vietnam (*Table 1*), 30 of which were derived from rubber trees (*Hevea brasiliensis*), four from papaya (*Carica papaya*) and one each from *Ecdysanthera rosea*, wild mango (*Irvingia malayana*), white pumpkin (*Benincasa cerifera*) and sesame (*Sesamum indicum*). All isolates were purified to single spore cultures to ensure the genetic uniformity and cultured on potato dextrose agar (PDA, DIFCO Laboratories).

DNA Extraction

DNA was extracted from 5-day old fungal mycelia grown on PDA plates at room temperature. The mycelia were harvested by scraping the fungal colonies with a sterile glass slide. DNA extraction was performed using QIAGEN DNeasy Plant Mini Kits (Qiagen, Germany). The purity and the concentration of extracted DNAs were measured using a spectrophotometer (Dr 5000 – Hach, USA). After extraction, genomic DNA was stored at 4ºC and used as mother stock to prepare template DNA (10 ng/µL) prior to PCR.

PCR Amplification and Sequencing

Amplification of rDNA ITS was done using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as the forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as the reverse primer, which were described by White *et al*.[24]. PCR amplification was performed in a volume of 25 µL reaction containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of ITS1 primer, 1 µM of ITS4 primer, 0.5U Go Taq@ Flexi DNA Polymerases and 10 ng of template DNA. The amplification was conducted in a thermocycler with initial denaturation at 94ºC for 2 min, followed by 35 cycles of 20 s at 94ºC, 30 s at 53ºC, 30 s at 72ºC and a final extension step at 72ºC for 5 minutes. The amplification was repeated thrice.

PCR products from three replications of each isolate were accumulated and then sequenced by a commercial sequencing service (First BASE Laboratories Sdn. Bhd. Malaysia) using the primers ITS1 and ITS4 with BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems), which is based on Sanger's dideoxy sequencing technology.

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

No	Isolate	Location	Infected host	Collection year
1	CoryLK02	Lai Khe	LH 01/0131	2009
2	CoryLK22	Lai Khe	RRIV 3	2010
3	CoryLK23	Lai Khe	RRIV 4	2010
4	CoryLK24	Lai Khe	IAN 873	2010
5	CoryLK25	Lai Khe	LH 90/952	2010
6	CoryLK26	Lai Khe	Papaya	2010
7	CoryLK27	Lai Khe	LH 90/1094	2010
8	CoryLK28	Lai Khe	LH 98/241	2010
9	CoryLK29	Lai Khe	RRIV 3	2010
10	CoryLK30	Lai Khe	RRIV 2	2010
11	CoryLK31	Lai Khe	RRIV 3	2010
12	CoryLK32	Lai Khe	LH 94/062	2010
13	CoryLK33	Lai Khe	RRIV 1	2010
14	CoryLK34	Lai Khe	RRIV 2	2010
15	CoryLK35	Lai Khe	RRIV 3	2010
16	CoryLK36	Lai Khe	Wild mango	2009
17	CoryLK37	Lai Khe	*Ecdysanthera rosea*	2009
18	CoryLK38	Lai Khe	White pumpkin	2009
19	CoryDT01	Dau Tieng	RRIV 3	2010
20	CoryDT02	Dau Tieng	RRIV 2	2010
21	CoryDT03	Dau Tieng	RRIV 4	2010
22	CoryDT04	Dau Tieng	RRIV 4	2010
23	CoryDT05	Dau Tieng	PB 260	2010
24	CoryDT06	Dau Tieng	RRIV 4	2010
25	CoryDT07	Dau Tieng	Sesame	2010
26	CoryTN02	Tay Ninh	RRIV 4	2010
27	CoryPG01	Phu Giao	RRIV 4	2010
28	CoryDP02	Dong Phu	Papaya	2010
29	CoryDP03	Dong Phu	RRIV 4	2010
30	CoryDP04	Dong Phu	RRIV 2	2010
31	CoryCC01	Cu Chi	Papaya	2010
32	CoryCC02	Cu Chi	Papaya	2010
33	CoryDN03	Dong Nai	RRIV 4	2010
34	CoryDN04	Dong Nai	RRIM 712	2010
35	CoryKT03	Kon Tum	RRIV 4	2010
36	CoryKT04	Kon Tum	PB 260	2010
37	CoryPK01	Gia Lai	LH 88/732	2009
38	CoryQN02	Quang Nam	RRIV 4	2009

Phylogenetic Analysis

The sequence of each isolate was refined using BioEdit software version 7.0.9.0[25], where the sequence obtained from the reverse primer ITS4 was transformed to reverse complement and aligned with the sequence obtained from forward primer ITS1 for editing. The refined rDNA ITS sequences of 38 isolates were aligned using the Clustal W. Multiple alignment which is bundled in the BioEdit software to explore the nucleotide variability among isolates. BLAST[26] alignment was conducted with two representative sequences of cluster 1 and 2 obtained from the alignment to identify the species of the studied isolates.

ISSR Analysis

In order to analyse the genetic relationship between isolates using ISSR markers, a total of eight ISSR primers were used for PCR amplification. The properties of these primers including names, sequences and annealing temperatures were shown in *Table 2*.

PCR amplification of ISSR loci was carried out in a 20 µL reaction volume containing 1 × PCR buffer [10 mM Tris-HCl (pH 8.8 at 25ºC), 50 mM KCl, 50 mM Triton X-100], 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.75 µM of ISSR primer, 1.5 U Go Taq@ Flexi DNA Polymerases and 20 ng of template DNA. The amplification was performed in a Thermocycler (PTC 2000, MJ Research) with initial denaturation at 94ºC for 5 min, followed by 35 cycles of 30 s at 94ºC, 1 min at 51ºC – 55ºC (depending on primers, *Table 2*), 2 min at 72ºC and the final extension at 72ºC for 10 minutes. The amplified products were size-separated in 2% agarose gel in 1 × TAE buffer at 70 V for 2 h at room temperature. Gels were stained with ethidium bromide, visualised under UV light and photographed using a gel documentation system (GelDoc-It 310 Imaging System, UVP). The sizes of amplified DNA fragments were estimated by comparison with the Bench Top 1 kb DNA ladder (Promega).

The stable amplified DNA fragments were scored as 1 for presence and 0 for absence of DNA band. The binary matrix data were then

TABLE 2. LIST OF ISSR PRIMERS, THEIR SEQUENCES, ANNEALING TEMPERATURES AND DNA POLYMORPHISM USED TO DIFFERENTIATE *CORYNESPORA CASSIICOLA* ISOLATES

Primer	Sequence (5' – 3')	Annealing t^o	Amplified DNA fragments	Polymorphic fragments
UBC 826	ACACACACACACACACC	55ºC	18	17
UBC 828	TGTGTGTGTGTGTGTGA	52ºC	7	7
UBC 835	AGAGAGAGAGAGAGAGYC	54ºC	12	11
UBC 840	GAGAGAGAGAGAGAGAYT	54ºC	2	1
UBC 850	GTGTGTGTGTGTGTGTYC	55ºC	11	11
Mj3	GTGCCTGTGCCTG	51ºC	14	13
Mj4	GCCAACGCCAACG	51ºC	14	14
Mj5	CCGTCACCGTCAA	51ºC	10	9
	Total number of fragments		88	83

Nomenclature: Y= C/T
UBC ISSR Primers designed by University of British Columbia, Vancouver, Canada.
Mj ISSR primers designed from the microsatellite regions of *Ganoderma* spp.

used for analysis of genetic similarity index and for genetic differentiation among the isolates. Cluster analyses were performed from Nei and Li's similarity matrix using unweighted paired group method with arithmetic mean (UPGMA) with 1000 bootstrapped replications using FreeTree software version 0.9.1.50[27]. A dendrogram was produced using Treeview software version 1.6.6[28].

Pathogenicity Tests

Based on phylogenetic analysis using ITS rDNA sequences, four *C. cassiicola* isolates that represented two genetic groups and different host sources in Vietnam were selected to test their pathogenicity on rubber trees, papaya and white pumpkin using detached leaf assay and artificial inoculation in greenhouse condition. The inoculant was prepared as the spore suspension of *C. cassiicola* from the 10-day old cultures on PDA plates in the sterile distilled water containing 0.05% Tween 20. The resulting suspension was then filtered through two layers of gauze and adjusted to 2×10^3 spores/mL for detached leaf assay and 7×10^3 spore/mL for artificial inoculation on plantlets in greenhouse.

Regarding detached leaf assay, the rubber leaflets were collected at the light green stage, *i.e.* 12-15 days old after budburst and the papaya and white pumpkin leaflets were collected at the early mature stage. The detached leaflets were placed in moist plastic boxes with the abaxial surfaces facing upward. The inoculation was done by dropping 10 µL of *C. cassiicola* spore suspension (2×10^3 spores/mL) onto the leaflets with eight spots on each rubber leaflet or 24 spots on each papaya or white pumpkin leaflet. The leaflets were then kept under fluorescent light 12 h/day in an air conditioned room (25–26ºC) for six days. The development of infections was scored after six days of the inoculation using the assessment method adopted from Ismail and Jeyanayagi[29] with modification by Ismail as described by Nghia *et al*.[21]. The experiments were arranged in completely randomised design (CRD) in three replicates. Each replicate consisted of three rubber detached leaflets or one papaya leaflet or one white pumpkin leaflet. Percentage disease intensity (PDI) in each replicate was calculated using *Equation 1*:

$$\text{PDI \%} = \frac{\text{Sum of numerical ratings}}{\text{Number of droplet observed} \times \text{Maximum disease grade}} \times 100 \quad \ldots 1$$

The data of PDI values were transformed to arsine square root and subjected to analysis of variance (ANOVA) and Duncan's multiple range test using SAS software version 9.0.

Meanwhile, for pathogenicity test on plantlets in green house condition, the experiments were laid out in randomised complete block design (RCBD) with five replicates. Each replicate consisted of two rubber plantlets, two papaya seedlings and three to four white pumpkin seedlings. The whole leaf canopy of the uniform plantlets was sprayed with the spore suspension of *C. cassiicola* (7×10^3 spores/mL). After 24 h of inoculation, the humidity system was used to provide good condition for the disease to develop. The infection score was recorded after 10 days of the inoculation based on lesions on leaflets as 0: No lesions on the leaflet, 1: A few small lesions up to 2-3 spots which occupies less than 1/8 leaf area, 2: A few lesions account for 1/8 leaf area (12.5%), 3: The lesions account for 1/4 leaf area (25%), 4: The lesions account for 1/2 leaf area (50%) and 5: The lesions account for more than 50% of the leaf area. Percentage disease intensity (PDI) in each replicate was calculated using the same formula as mentioned earlier in this report and disease severity level was described based on the average infection score

(AIS) (*Table 3*) which was calculated using *Equation 2*:

$$AIS = \frac{\text{Sum of numerical infection scores}}{\text{Total number of plants observed}} \quad \ldots 2$$

RESULTS

Ribosomal DNA-ITS (rDNA ITS) Sequence Analysis

PCR amplifications of total genomic DNA using primer pair ITS1–ITS4 produced a single PCR product which included ITS1-5.8S rDNA-ITS2 region in all studied isolates. DNA sequencing confirmed that the DNA fragments generated from all isolates were equal in length with 559 bp (*Figure 1*). These sequences were submitted to the GenBank[30] with the assigned accession numbers from KF387577 to KF387614. Sequence alignment revealed that the rDNA ITS regions of all 38 isolates were identical with the exception of one single nucleotide polymorphisms (SNPs) detected in the ITS1 region. From the analysis, DNA sequences of 21 isolates collected from Lai Khe (CoryLK02, CoryLK22, CoryLK23, CoryLK24, CoryLK25, CoryLK27, CoryLK28, CoryLK29, CoryLK30, CoryLK31, CoryLK33, CoryLK34, CoryLK35), Dau Tieng (CoryDT02, CoryDT03, CoryDT06), Dong Phu (CoryDP03), Cu Chi (CoryCC01, CoryCC02), Dong Nai (CoryDN03) and Quang Nam (CoryQN02) had a Cytosine (C) residue at the 135th base pair while 17 isolates collected from Lai Khe (CoryLK26, CoryLK32, CoryLK36, CoryLK37, CoryLK38), Dau Tieng (CoryDT01, CoryDT04, CoryDT05, CoryDT07), Tay Ninh (CoryTN02), Phu Giao (CoryPG01), Dong Phu (CoryDP02, CoryDP04), Dong Nai (CoryDN04), Kon Tum (CoryKT03, CoryKT04) and Gia Lai (CoryPK01) have Thymine (T) residue at the same position (*Figure 2*). As such, the studied isolates were clearly grouped into two clusters based on the SNP found at 135th nucleotide. Cluster 1 consisted of 21 isolates and cluster 2 contained 17 isolates.

The BLAST search results of the nucleotide sequences of isolates CoryLK02 and CoryDP04 representing the isolates of clusters 1 and cluster 2, respectively, showed high similarity between these sequences and deposited sequences identified as *C. cassiicola* in NCBI GenBank database.

ISSR PCR Analysis

A total of 88 consistently amplified DNA bands were generated from eight ISSR primers, of which 94.3% were polymorphic (*Table 2*, *Figure 3*). Dendrogram produced from UPGMA analysis based on Nei and Li's coefficient divided 38 studied isolates into two main clusters (*Figure 4*). Cluster 1 included 21 isolates from Lai Khe (CoryLK02, CoryLK22, CoryLK23, CoryLK24, CoryLK25, CoryLK27, CoryLK28, CoryLK29, CoryLK30, CoryLK31, CoryLK33, CoryLK34, and CoryLK35), Dau

TABLE 3. DISEASE SEVERITY INDEX BASED ON AVERAGE INFECTION SCORES

AIS	Disease severity	Host reaction
0.0	No disease	Highly resistant
0.1 – 1.0	Slight infection	Mild
1.1 – 2.0	Moderate infection	Moderate
2.1 – 3.0	Severe infection	Severe
3.1 – 5.0	Very severe infection	Very severe

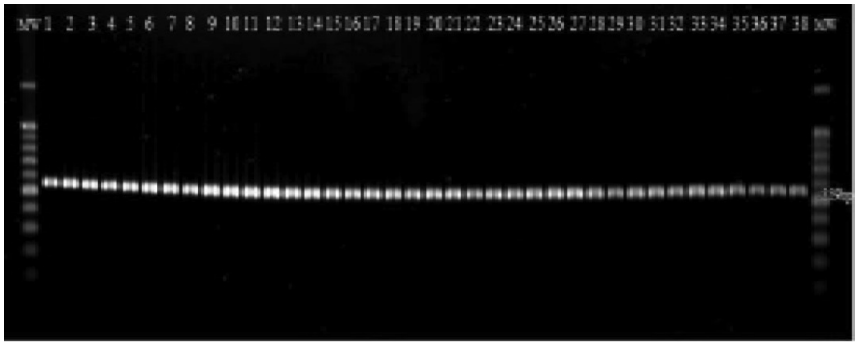

Figure 1. Gel electrophoresis of amplified products obtained from 38 C. cassiicola *isolates using ITS1 and ITS4 primers. The 1st lane (MW) is a 2-Log DNA Ladder 100 bps (Promega).*

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                                 130       140       150       160
                          ....|....|....|....|....|....|....|....|
01  CoryLK02   CCACAAACCCATTGCAGTACAAGAAGTACACGTCTGAACA
02  CoryLK22   ....................................................
03  CoryLK23   ....................................................
04  CoryLK24   ....................................................
05  CoryLK25   ....................................................
06  CoryLK26   ..............T.....................................
07  CoryLK27   ....................................................
08  CoryLK28   ....................................................
09  CoryLK29   ....................................................
10  CoryLK30   ....................................................
11  CoryLK31   ....................................................
12  CoryLK32   ..............T.....................................
13  CoryLK33   ....................................................
14  CoryLK34   ....................................................
15  CoryLK35   ....................................................
16  CoryLK36   ..............T.....................................
17  CoryLK37   ..............T.....................................
18  CoryLK38   ..............T.....................................
19  CoryDT01   ..............T.....................................
20  CoryDT02   ....................................................
21  CoryDT03   ....................................................
22  CoryDT04   ..............T.....................................
23  CoryDT05   ..............T.....................................
24  CoryDT06   ....................................................
25  CoryDT07   ..............T.....................................
26  CoryTN02   ..............T.....................................
27  CoryPG01   ..............T.....................................
28  CoryDP02   ..............T.....................................
29  CoryDP03   ....................................................
30  CoryDP04   ..............T.....................................
31  CoryCC01   ....................................................
32  CoryCC02   ....................................................
33  CoryDN03   ....................................................
34  CoryDN04   ..............T.....................................
35  CoryKT03   ..............T.....................................
36  CoryKT04   ..............T.....................................
37  CoryPK01   ..............T.....................................
38  CoryQN02   ....................................................
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Figure 2. Single nucleotide polymorphisms located at position 135 in ITS1 region of 38 C. cassiicola *isolates. The number on the top show the position of nucleotide counted from the 5' end of the sequence.*

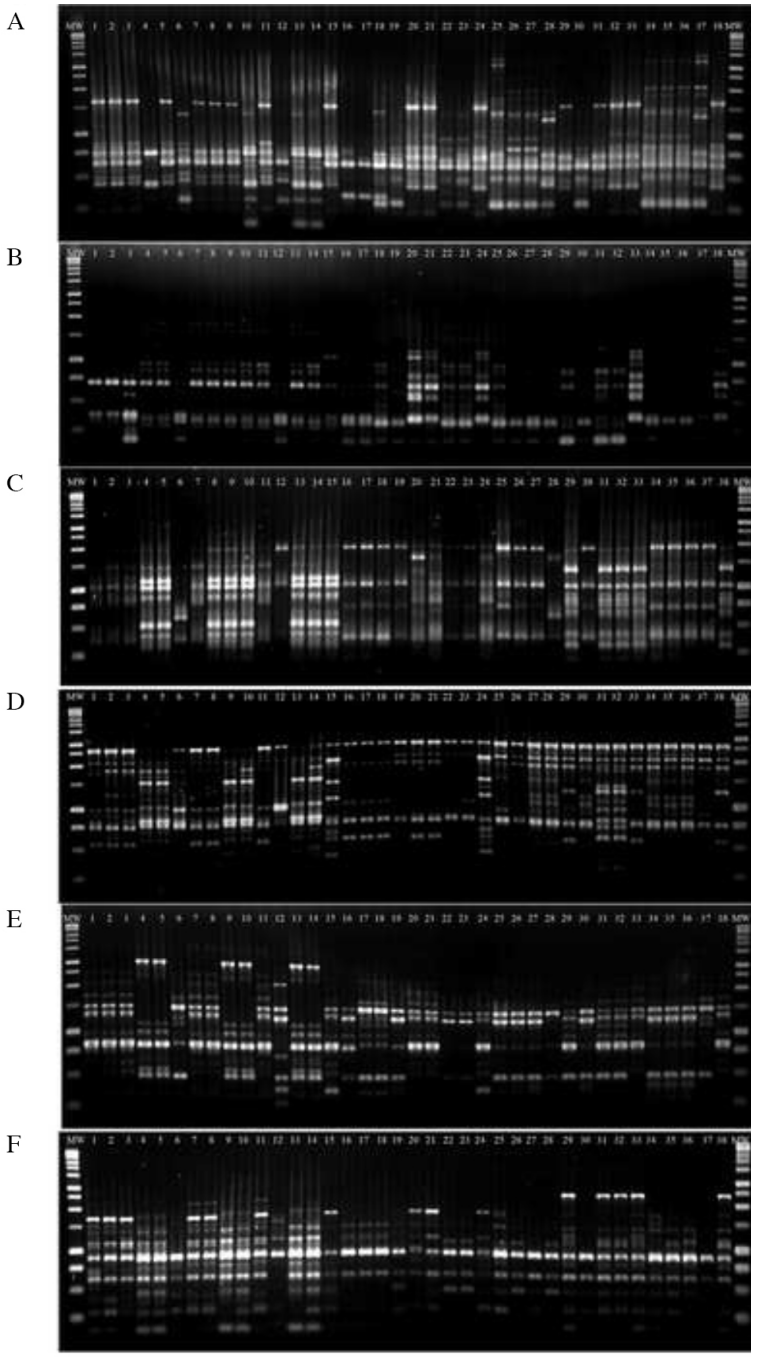

Figure 3. Gel electrophoresis of amplified products from C. cassiicola *genomic DNA obtained by 6 ISSR primers UBC 826 (A), UBC 835 (B), UBC 850 (C), Mj3 (D), Mj4 (E) and Mj5(F) using the ISSR-PCR technique. The 1st and the last lanes (MW) are 1 kb DNA ladder markers (Promega).*

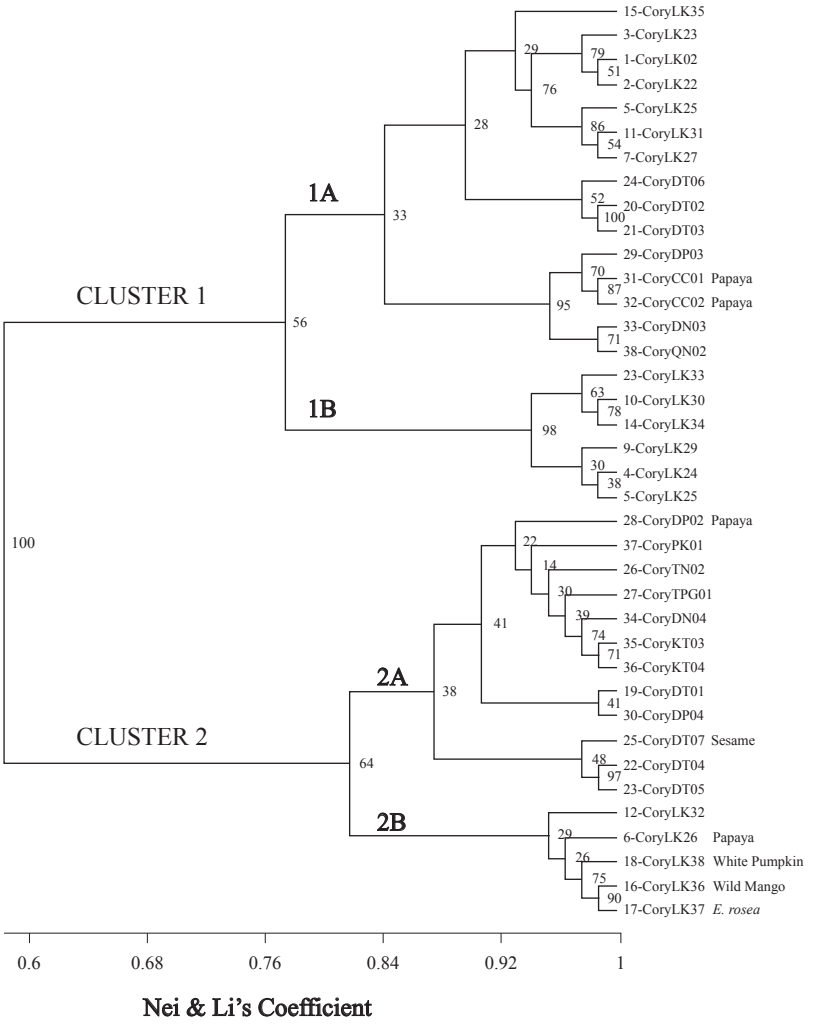

Figure 4. Dendrogram derived from UPGMA cluster analysis, using Nei & Li's coefficient based on 88 ISSR bands showing the genetic relationships among 38 C. cassiicola *isolates.*

Tieng (CoryDT02, CoryDT03, and CoryDT06), Dong Phu (CoryDP03), Cu Chi (CoryCC01, CoryCC02), Dong Nai (CoryDN03) and Quang Nam (CoryQN02). This cluster was split into 2 subclusters (1A, 1B). Subcluster 1A contained 15 isolates (CoryLK02, CoryLK22, CoryLK23, CoryLK27, CoryLK28, CoryLK31, CoryLK35, CoryDT02, CoryDT03, CoryDT06, CoryDP03, CoryCC01, CoryCC02, CoryDN03 and CoryQN02), subcluster 1B consisted of six isolates collected from Lai Khe. Cluster 2 comprised of 17 isolates and was also split into two subclusters (2A, 2B). Subcluster 2B included 5 isolates collected from Lai Khe (CoryLK26, CoryLK32, CoryLK36, CoryLK37, and CoryLK38), subcluster 2A included the remaining isolates from other areas (CoryDT01, CoryDT04, CoryDT05,

CoryDT07, CoryTN02, CoryPG01, CoryDP02, CoryDP04, CoryDN03, CoryKT03, CoryKT04 and CoryPK01). The bootstrap values for cluster 1 and 2 were 86% and 64%, respectively. The similarity index was calculated at 58% between the two clusters. The isolates in cluster 1 showed 77% similarity and cluster 2 exhibited 62% similarity. Members of each cluster derived from this analysis corresponded to those of the clusters generated from the analysis of rDNA ITS sequences obtained from the 38 studied isolates.

Pathogenicity Test

The pathogenicity of four representative isolates (CoryLK02, CoryPK01, CoryLK26, CoryLK38) on detached leaflets of seven rubber clones (PB 255, PB 260, RRIV 1, RRIV 3, RRIV 4, RRIV 5, RRIV 124), papaya and white pumpkin after six days of inoculation differed from each other, resulting in no visible lesions or small dark discolouration below the droplets or fully prominent large lesions with or without mycelium growth. The average infection scores and the percent disease intensity (PDI) were shown in *Table 4*.

Isolate CoryLK02 caused severe infection on rubber clones such as PB 255, RRIV 1, RRIV 3, RRIV 4 and RRIV 5 with AIS up to 4.0 and PDI up to 100%. This isolate caused moderate infection on clones RRIV 124 and PB 260 with AIS of 2.8 and 2.9, PDI of 69.4% and 73.6%, respectively. This isolate caused slight infection on white pumpkin and moderate infection on papaya with AIS of 1.4 and 2.3, PDI of 36.1% and 58.3%, respectively. The Duncan's multiple range test separated the studied clones/hosts into four groups based on PDI and there were significant differences between the groups.

Isolate CoryPK01 caused severe infection on clone RRIV 4 with AIS value of 3.1, moderate infection on RRIV 1 and RRIV 3 (AIS values of 2.5 and 2.8, respectively), slight infection on clones PB 255, RRIV 5, RRIV

TABLE 4. PATHOGENICITY OF THE REPRESENTATIVE *C. CASSIICOLA* ISOLATES ON SEVEN RUBBER CLONES, PAPAYA AND WHITE PUMPKIN USING DETACHED LEAF ASSAY

Clones/hosts	Isolates							
	CoryLK02		CoryPK01		CoryLK26		CoryLK38	
	AIS	PDI (%)	AIS	PDI (%)	AIS	PDI (%)	AIS	PDI (%)
PB 255	4.0	100.0^{a}	1.6	39.9^{c}	2.0	49.7^{cd}	3.8	96.2^{b}
PB 260	2.8	69.4^{b}	0.8	20.1^{d}	3.7	91.3^{a}	3.6	90.3^{b}
RRIV 1	4.0	100.0^{a}	2.5	63.5^{b}	3.3	83.7^{ab}	4.0	100.0^{a}
RRIV 3	4.0	100.0^{a}	2.8	75.7^{a}	2.5	62.5^{bcd}	4.0	100.0^{a}
RRIV 4	4.0	100.0^{a}	3.1	76.4^{a}	3.6	88.9^{a}	4.0	100.0^{a}
RRIV 5	4.0	100.0^{a}	1.8	45.5^{c}	0.1	1.4^{e}	0.3	7.6^{d}
RRIV 124	2.9	73.6^{b}	1.8	44.8^{c}	2.7	67.7^{abc}	3.8	94.1^{b}
Papaya	2.3	58.3^{c}	0.0	0.0^{e}	1.4	35.1^{d}	1.2	29.2^{c}
White pumpkin	1.4	36.1^{d}	0.0	0.0^{e}	0.0	0.0^{e}	1.1	27.8^{c}
CV (%)	4.39		7.91		20.74		8.44	

AIS: average infection score; PDI (%): percent disease intensity (%).
Values in the same column followed with the same superscript letter are not significantly different at $P<0.05$ by Duncan's multiple range test.

124 (AIS ranged from 1.3 to 2.0) and very slight infection on PB 260. This isolate caused no symptoms on leaves of papaya and white pumpkin. Analysis of means of PDI values using Duncan's multiple range test separated the clones/hosts into five groups, the clones/hosts in the same group were not significantly different but they were significantly different from the clones/hosts in the other groups. Regarding rubber clones, they were clustered into four groups; two clones RRIV 3 and RRIV 4 with the respective PDI values of 75.7% and 76.4% were clustered in group 1. Group 2 only consisted of RRIV 1 with PDI value of 63.5%, while group 3 consisted of PB 255, RRIV 124 and RRIV 5. It is worth noting that PB 260 was only slightly infected and assigned in one distinct group (group 4).

Isolate CoryLK26 caused severe infection on 3 clones RRIV 1, RRIV 4 and PB260 with AIS ranging from 3.3 to 3.7. Two clones RRIV 3 and RRIV 124 were moderately infected, but RRIV 5 and PB 255 were only infected at a slight or moderate level. Although CoryLK26 was isolated from papaya, it only caused slight infection on papaya leaves (AIS value of 1.4 and PDI value of 35.1%) and it was not virulent to white pumpkin leaves.

Isolate CoryLK38 caused severe infection on all rubber clones, *i.e*. PB 260, PB 255, RRIV 124, RRIV 1, RRIV 3 and RRIV 4, with AIS values ranging from 3.6 to 4.0, except that RRIV 5 was infected at negligible level (AIS value of 0.3). Although CoryLK38 was isolated from white pumpkin, it only caused slight infection on the leaves of this host (AIS value of 1.1 and PDI value of 27.8%). This isolate also caused slight infection on papaya leaves (AIS value of 1.2 and PDI value of 29.2%). The Duncan's multiple range test separated the clones/hosts into three groups based on PDI values which were significantly different among the clones/hosts.

In greenhouse condition, after 10 days of inoculation with spore suspensions of 4 *C. cassiicola* representative isolates, seven rubber clones, papaya and white pumpkin displayed none to distinct symptoms. PDI values ranged from 0.0 to 57.6% and AIS ranged from 0.0 to 2.9 (*Table 5*). All isolates were virulent to the tested rubber clones. Similar to the result of detached leaf assay, isolate CoryLK02 caused slight infection on papaya and white pumpkin, but the isolate CoryPK01 did not cause any symptoms on these hosts. Isolate CoryLK26 infected papaya and all of the rubber clones in this experimental conditions. CoryLK38 not only infected white pumpkin but also rubber trees and papaya.

Isolate CoryLK02 caused severe disease on rubber clones RRIV 3 and RRIV 4 with AIS values of 2.5 and 2.9 and PDI values of 50.0 and 57.6%, respectively. This isolate caused moderate infection on PB 255 and slight infection on RRIV 124, PB 260, RRIV 1 and RRIV 5, of which AIS ranged from 0.6 to 0.8 and PDI ranged from 11.6% to 15.6%. In addition, this *C. cassiicola* isolate also slightly infected papaya and white pumpkin, of which AIS were 0.3 and 0.1, PDI were 6.0 and 2.8, respectively. The Duncan's multiple range test clustered the clones/hosts into five groups based on PDI values with significant differences between the groups.

Isolate CoryPK01 caused severe disease on clones RRIV 3 and RRIV 4 (AIS of 2.3 and 2.4, respectively) and slight disease on the remaining rubber clones (AIS less than 1.0). No disease symptoms were recorded on papaya and white pumpkin, suggesting that this *C. cassiicola* isolate were avirulent to papaya and white pumpkin. The Duncan's multiple range test based on PDI values separated the clones/hosts into several groups; group 1 included RRIV 4 and RRIV 3, of which PDI values were higher than those of the other groups,

TABLE 5. THE INFECTION LEVEL OF FOUR ISOLATES OF *C. CASSIICOLA* ON SEVEN RUBBER CLONES, PAPAYA AND WHITE PUMPKIN SEEDLINGS IN GREENHOUSE CONDITION

Clones/hosts	Isolates							
	CoryLK02		CoryPK01		CoryLK26		CoryLK38	
	AIS	PDI (%)	AIS	PDI (%)	AIS	PDI (%)	AIS	PDI (%)
PB255	1.2	24.0^{b}	0.8	16.0^{b}	0.4	8.0^{c}	0.6	12.8^{b}
PB260	0.7	13.6^{cd}	0.3	6.8^{bc}	0.3	5.6^{c}	0.4	7.6^{bc}
RRIV1	0.8	15.6^{c}	0.4	8.8^{bc}	0.4	8.0^{c}	0.4	8.8^{b}
RRIV3	2.5	50.0^{a}	2.4	48.0^{a}	1.9	37.2^{a}	1.5	30.4^{a}
RRIV4	2.9	57.6^{a}	2.3	45.6^{a}	1.8	35.6^{a}	2.0	39.2^{a}
RRIV5	0.8	15.6^{c}	0.3	6.4^{c}	0.4	8.0^{c}	0.4	8.4^{b}
LH90/952	0.6	11.6^{c}	0.4	8.4^{bc}	0.4	8.4^{c}	0.5	9.6^{b}
Papaya	0.3	6.0^{d}	0.0	0.0^{d}	0.7	14.8^{b}	0.1	1.6^{c}
White pumpkin	0.1	2.8^{e}	0.0	0.0^{d}	0.0	0.0^{d}	0.3	6.4^{bc}
CV (%)	18.41		31.09		18.14		30.38	

AIS: average infection score; PDI (%): percent disease intensity (%).
Values in the same column followed with the same superscript letter are not significantly different at $P<0.05$ by Duncan's multiple range test.

and there were significant differences among the groups.

Isolate CoryLK26 infected all of the tested rubber clones with the most severity on RRIV 4 and RRIV 3, of which AIS ranged from 1.8 to 1.9, and slight infection on other clones, of which AIS ranged from 0.3 to 0.4. Although CoryLK26 was isolated from papaya, it only caused slight infection on this host with AIS of 0.7 and PDI of 14.8%. This isolate did not infect white pumpkin.

Isolate CoryLK38 also infected all of the rubber clones used in this study with slight infection on PB 260, RRIV 1, RRIV 5, PB 255, RRIV 124 and moderate or severe infection on RRIV 3 and RRIV 4. The symptoms of the disease were recorded on papaya and white pumpkin plants at slight infection level with AIS ranging from 0.1 to 0.3 and PDI ranging from 1.6% to 6.4%. The PDI values among clones/hosts tested were not significantly different except RRIV 3 and RRIV 4.

DISCUSSION

The ITS consisted of two variable regions (ITS 1 and ITS2) that are located within the rDNA repeats between the highly conserved small subunit (5.8S) and the large subunit rRNA genes. Ribosomal DNA internal transcribed spacer sequences were known to evolve quickly and therefore the ITS region is useful for molecular characterisation in fungi due to its similarity within and variability between fungal species. The difference in sizes and sequences might be detected within a species. In this study, the primers ITS1 and ITS4 were aimed to amplify a variable region between the 3' end of the small subunit (18S) rDNA and the 5' end of the large (28S) rDNA which includes the 5.8S rDNA gene flanked by two internal transcribed spacers. The equal length of rDNA ITS fragments amplified in all 38 isolates indicates that intraspecific variation within isolates cannot be detected if using only the PCR approach as it was also reported in a previous study conducted in *C. cassiicola*[20]. Sequence analysis of rDNA ITS revealed one

single nucleotide polymorphism (SNP) at the 135th nucleotide (C/T). Nucleotide variation at this position was also reported by Nghia *et al.*[22] and Dixon *et al.*[23]. According to Nghia *et al.*[22], nucleotide variation at this position helped to differentiate *C. cassiicola* isolates into two different races. Comparison of the finding from this study and that from Nghia *et al.*[22] implied that there are at least two different races of *C. cassiicola* existing in Vietnam. *C. cassiicola* isolates possessing C residue at position 135 belong to Race 1, which was identified to severely infect RRIM 600 and other earlier clones[30], and *C. cassiicola* isolates possessing T residue at position 135 belong to Race 2, which was identified to infect RRIM 2020 and newer clones[30]. However, pathogenesis test on the mentioned clones should be conducted before any further conclusions on the pathological characteristics are made.

Molecular markers have been widely employed to determine the genetic characteristics of fungi, plants and animals. The invention of PCR led to the development of faster and inexpensive molecular markers. Several successful uses of PCR-based techniques have been reported for the identification of fungal pathogen at a taxonomic level lower than the species[31]. Randomly Amplified Polymorphic DNA (RAPD) revealed significant polymorphism among groups of *C. cassiicola* isolates. Several earlier works have reported correlation between the RAPD groups and the features of the isolates *e.g*. pathogenicity, geographical origin, host plant genotype from which the isolate was collected[15,20] but some others have found no correlation[14,18]. ISSR is another PCR based technique that is similar to RAPD technique except that the ISSR primer sequences are longer and are designed from microsatellite regions. Therefore, the annealing temperatures used in PCR reactions are higher than those used for RAPD markers, which could lead to higher consistency of the PCR products. The advantage of the ISSR technique lies in its effective multilocus markers used for diversity analysis, fingerprinting and genome mapping. They are easy to employ and are highly reproducible compared with other techniques such as RAPD and no prior sequence knowledge is required[32]. The ISSR have been successfully applied to study the genetic diversity of pathogenic fungi, *e.g. Beauveria bassiana*[33]. This study showed that 94.3% of the bands generated by ISSR markers were polymorphic, which reflects the high level of genetic variation that exists among the different isolates. The low similarity index value (58%) between the two clusters showed high genetic variability between the two groups of isolates. Similar results were reported by Nghia *et al.*[21] when ISSR markers were used to characterise 21 *C. cassiicola* isolates collected from different regions of Malaysia. Interestingly, cluster analysis using rDNA ITS sequences and UPGMA cluster analysis using profiles generated by ISSR markers resulted in the same number of groups and the members in each group. In this study, the fact that *C. cassiicola* isolates were isolated from a wide range of rubber clones and some of other hosts grown in different regions of Vietnam suggested that there is no correlation between the genetic groups of *C. cassiicola* and the host origins or geographical regions. The distribution of these two races of *C. cassiicola* in all geographical regions could result from the dissemination of the spores or the importation of the diseased planting materials.

Pathogenesis test plays an important role in the study of host-pathogen interactions, of which clear understanding helps to improve the management of the disease caused by the studied pathogen. Shimomoto *et al.*[19] stated that pathogenicity tests are the keys in the identification of the species and the races of fungus. Previous studies revealed the

differences of the virulence of *C. cassiicola* isolates from one host on another host, which contributed to the formation of different *C. cassiicola* pathogenicity groups. Some *C. cassiicola* isolates showed virulence on a wide range of host whereas others exhibited host specificity[34–35]. So far, many pathogenicity groups of *C. cassiicola* have been detected and isolates of a pathogenicity group might show different levels of virulence on the same hosts[19,20,23,35]. It was suggested that grouping isolates based on their original host is problematic for predicting pathogenicity and genetic relatedness[19]. In this study, different demonstrations of pathogenicity on white pumpkin and papaya indicated a lack of correlation between phylogenetic data and pathogenicity of *C. cassiicola*. This could be due to the pathogenicity of the individual isolates depending on the specificity and the susceptibilities of the host plants. Pernezny and Simone[36] reported that many races of *C. cassiicola* from different hosts have been found but are not always cross infective to other host. Onesirosan *et al.*[35] observed that *C. cassiicola* isolate from *Calopogonium mucunoides*, *Synedrella nodiflora*, cucumber, soybean and tomato were highly virulent on tomato and egg plants but only moderately virulent on sesame and cotton. Cutrim and Silva[34] tested the pathogenicity of two isolates of *C. cassiicola* obtained from tomato on several hosts, the host plants tested reacted differently to the pathogen but most of them were susceptible to both isolates except Barbados cherry and papaya. Poltronieri *et al.*[37] inoculated the *Malpighia glabra* leaves using *C. cassiicola* isolates obtained from several hosts, the isolates from *Rhododendrum* sp., tomato, squash and *M. glabra* induced typical symptoms on *M. glabra* leaves but the isolates from *Piper hispidinervium* and papaya failed to induce the symptoms. In another study, 15 *C. cassiicola* isolates collected from several hosts were inoculated on 12 different plant species, the difference in host range was found among isolates and the susceptibility of the hosts to different isolates was also dissimilar[38]. In Malaysia, it was reported that *C. cassiicola* isolates from rubber were host specific and did not infect chilli, cocoa, papaya, tomato, lettuce, soybean and oil palm[39–40]. In Indonesia, Suwarto *et al*.[41] reported that *C. cassiicola* isolate obtained from papaya caused disease on some rubber clones and *C. cassiicola* isolates derived from rubber did not infect soybean and cassava. Importantly, the capability of *C. cassiicola* from other host plants, *e.g*. papaya and white pumpkin, to infect rubber trees found in this study posed a warning in using these host plants as intercropping plants in rubber plantations since such conditions favour the interference and cross infection between the host plants, which lead to the risk of disease outbreaks.

CONCLUSION

ISSR markers proved useful to distinguish the genotype groups of the fungus while rDNA ITS sequence markers could not only identify, but also infer the genotype groups of this fungus, thus, correlation between these molecular markers has been found. This study also confirmed that at least two distinct groups of *C. cassiicola* infect rubber trees and other hosts in Vietnam and it appears to be no correlation between phylogenetic groups of *C. cassiicola* and geographical regions or host origins or pathogenicity. It can be inferred from this study that *C. cassiicola* isolated from rubber trees can cause disease on other hosts and *vice versa*. This result revealed that *Corynespora* leaf disease on rubber trees can possibly be caused *via* the cross infection by *C. cassiicola* from another host in nature.

Date of receipt: June 2014
Date of acceptance: August 2014

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