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Cassiicolin Genes among Corynespora cassiicola *Isolates from Rubber Plantations in Malaysia*

SITI SHUHADA SHUIB[*], M. DEON[**,***], MURNITA MOHAMAD MAHYUDDIN[*], AZIMAH IZHAR[*], B. FUMANAL[**], E. SUNDERASAN[*#] AND V. PUJADE-RENAUD[**,***#]

Corynespora cassiicola *isolates are periodically sourced from infected stands in rubber plantations throughout Malaysia. The severity of* C. cassiicola *infection varies among the clonally propagated* Hevea brasiliensis*. In this study, 26* C. cassiicola *isolates collected from 1988 until 2006 were analysed for the gene encoding cassiicolin – an important fungal effector involved in the* Corynespora *leaf fall disease.* Cassiicolin *gene was successfully amplified by polymerase chain reaction (PCR) from 13* C. cassiicola *isolates. Deduced protein sequences revealed that 12 isolates harbour the* Cas5 *gene while one isolate (CKT05D) contains the* Cas4 *gene. The other 13 isolates in which no* Cas *gene was detected were classified as* Cas0*. PCR amplifications were also performed on all 26 isolates using primers specific to the ITS1-5.8S-ITS2 region of ribosomal DNA, the random hyper variable loci ga4 and caa5, and the actin locus act1. A phylogenetic analysis performed on the 26 isolates using four loci (rDNA ITS, caa5, ga4 and act1) revealed three clusters. Cluster 1 encompasses all* Cas5 *isolates plus two* Cas0 *isolates, CSD1 and CBPP2. Cluster 2 is represented by the single* Cas4 *isolate (CKT05D) and clusters 3 groups all the other* Cas0 *isolates. When placed in a previously described phylogenetic tree of* C. cassiicola *isolates from various geographical origins and hosts, clusters 1 and 3 fell in clades B4 and A4 respectively. However, CKT05D (Cluster 2) was not placed in a highly supported clade. A detached* Hevea *leaf assay was performed on six clones with a selection of four* C. cassiicola *isolates originating from the different clusters that showed varying degree of infectivity. Interestingly, severe necrotic lesion was discerned in most of the clones inoculated with CKT05D (toxin class* Cas4*) while the other isolates caused moderate to mild infection on all the tested clones.*

Keywords: *Corynespora cassiicola*; *Hevea brasiliensis*; toxin class; PCR; nucleotide and protein sequence characterisation; detached leaf assay

Corynespora cassiicola (Berk. & Curt.) is a fungal pathogen that infects more than 530 plant species from 380 genera, including a number of economically important ornamental plants and perennial crops like *Hevea brasiliensis*[1]. *C. cassiicola* causes severe leaf fall disease in *H. brasiliensis* plantation in many rubber producing countries. The fungus

[*]Rubber Research Institute of Malaysia, Malaysian Rubber Board, P.O. Box 10150, 50908 Kuala Lumpur, Malaysia.
[**]Clermont-Université, Université Blaise Pascal, UMR 547 PIAF, BP 10448, 63000 Clermont-Ferrand, France.
[***]CIRAD-UMR AGAP, F-63000 Clermont-Ferrand, France.
[#] Corresponding authors (e-mail: sunderasan@lgm.gov.my; valerie.pujade-renaud@cirad.fr)

predominantly infects the leaves and to a lesser extent, the leaf stalks and twigs of the rubber tree[2,3]. In Malaysia, early findings of infection were confined to nutrient deficient rubber seedlings and clones in budwood nurseries. The leaf fall disease however reached an epidemic stage in 1975 and has since become widespread in rubber plantations in many localities[4,5]. The increases in incidence of the disease and the clone-specific infectivity of *C. cassiicola* have been attributed to the emergence of different physiological races of the fungus[4,6].

Genetic diversity among *C. cassiicola* isolates obtained from rubber plantations has been reported[7–13] in relation with the host range of cultivated *Hevea*. While the secreted toxin cassiicolin was shown to be an important effector in some virulent strains[14,15], reports on new variants of cassiicolin are also emerging. So far, six cassiicolin isoforms (*Cas1* to *Cas6*) have been identified from isolates of various hosts and geographical origins[12,16]. Deon *et al*.[12] proposed a classification of the isolates based on the toxin class (*i.e*. the cassiicolin gene form) on one hand, and on the other hand the overall phylogenetic structure (established from the sequence alignment of four combined *loci*). A relative concordance was observed between the toxin classes and the phylogenetic groups although some isolates with no detectable cassiicolin gene classified as *Cas0*, were found in almost all the genetic groups.

The present study is aimed at investigating the genetic polymorphism of cassiicolin genes detected by PCR among *C. cassiicola* isolates obtained from rubber plantations in Malaysia in relation to the phylogenetic structure (determined by alignment of the four combined *loci i.e*. ribosomal DNA ITS, caa5, ga4 and act1). In addition, leaf bioassays were performed with selected *C. cassiicola* isolates to tentatively relate the toxin class and/or phylogenetic group to the pathogenicity profile.

MATERIALS AND METHODS

Fungal Isolates Culture Preparation

The *C. cassiicola* cultures were prepared by placing ten mycelial plugs taken from 10-day-old culture into Erlenmeyer flasks containing 200 mL of potato sucrose broth. The contents of the flasks were kept swirling at 150 rpm with orbital rotation at 28ºC for seven days. The growing mycelium was filtered through several layers of muslin cloth, washed with distilled water, and immediately immersed into liquid nitrogen. The remaining liquid was extracted from the mycelium using a freeze dryer. The mycelium was then kept in freezer at -80ºC. The genomic DNA extraction was performed using the DNeasy Plant Mini Kit (QIAGEN, CA) from 100 mg of lyophilised mycelium. The concentration of the fungal genomic DNAs was estimated by measuring optical density (A) at 260 nm. The quality of the genomic DNA samples was ascertained by electrophoresis on 0.8% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen™, CA). The *C. cassiicola* isolates used in this study are listed in *Table 1*.

Molecular Screening *via* PCR and Cloning of Cassiicolin

PCR amplification was performed on 26 *C. cassiicola* isolates using C1000™ Thermal Cycler (Bio-Rad, CA). The denaturation temperature was set at 94ºC for 45 s, annealing temperature at 52ºC for 45 s and extension temperature at 72ºC for 45 seconds. The reactions were programmed for 30 cycles. The primers used were designed from the flanking regions of the *Cas5* cassiicolin gene of the Malaysian CSB6 isolate[12]

(*Table 2*). They are able to amplify *Cas3*, *Cas4* and *Cas5* full length genes under low stringency conditions (data not shown). Other primers aimed at amplifying a partial sequence from the *Cas1*, *Cas2* or *Cas6* genes were also tested, without success. The PCR mixture contained 1X Green GoTaq® Promega, WI 2X DNA polymerase, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of PCR primers and 50 ng template of fungal genomic DNA. The amplification products were separated and on 2% agarose gel stained with SYBR® Safe DNA (Invitrogen™, CA). All PCR products were sequenced directly.

TABLE 1. *CORYNESPORA CASSIICOLA* ISOLATES OBTAINED FROM RUBBER PLANTATIONS THROUGHOUT MALAYSIA THAT WERE USED IN THE STUDY

No	*C. cassiicola* isolate	Host *Hevea* clone	Locality of collection	Year of collection	Genetic group	Toxin class
M1	CLN4	RRIM 2020	Lanchang, Pahang	1997	A4	*Cas0*
M2	CLN9	RRIM 2020	Lanchang, Pahang	1997	A4	*Cas0*
M3	CLN11	RRIM 2020	Lanchang, Pahang	1997	A4	*Cas0*
M4	CLN16	RRIM 2020	Lanchang, Pahang	1997	A4	*Cas0*
M5	CLN23	RRIM 2020	Lanchang, Pahang	2000	A4	*Cas0*
M6	CTD1	RRIM 2001	Tok Dor, Terengganu	1999	A4	*Cas0*
M7	CTD2	RRIM 2008	Tok Dor, Terengganu	1999	A4	*Cas0*
M8	CBS6	RRIM 2020	Bintulu, Sarawak	2003	A4	*Cas0*
M9	C68-6	RRIM 600	Sungai Buloh, Selangor	2006	B4	*Cas5*
M10	C68-8	RRIM 600	Sungai Buloh, Selangor	2006	B4	*Cas5*
M11	CSB3	RRIM 600	Sungai Buloh, Selangor	1988	B4	*Cas5*
M12	CSB5	RRIM 2001	Sungai Buloh, Selangor	1998	B4	*Cas5*
M13	CSB6	RRIM 2009	Sungai Buloh, Selangor	1998	B4	*Cas5*
M14	CSB11	RRIM 931	Sungai Buloh, Selangor	1998	B4	*Cas5*
M15	CSB15	RRIM 2020	Sungai Buloh, Selangor	1999	A4	*Cas0*
M16	CSB16	RRIM 600	Sungai Buloh, Selangor	1999	B4	*Cas5*
M17	CBN5	RRIM 2020	Banggol Nyor, Terengganu	2005	A4	*Cas0*
M18	CLKK1	RRIM 2025	Kuala Kangsar, Perak	2005	A4	*Cas0*
M19	CSD1	RRIM 901/ PR261/PB260	Sungai Durian, Perak	1996	B4	*Cas0*
M20	CBPP2	RRIM 600	Balik Pulau, Pulau Pinang	2006	B4	*Cas0*
M21	CKT1	RRIM 600	Kota Tinggi, Johor	1998	B4	*Cas5*
M22	CSB05A	BA 46	Sungai Buloh, Selangor	2005	B4	*Cas5*
M23	CSB05 B	BA 47	Sungai Buloh, Selangor	2005	B4	*Cas5*
M24	CKT05C	PB367	Kota Tinggi, Johor	2005	B4	*Cas5*
M25	CKT05D	RRIM 928	Kota Tinggi, Johor	2005	-	*Cas4*
M26	CKT05F	RRIM 2005	Kota Tinggi, Johor	2005	B4	*Cas5*

Footnote

The *C. cassiicola* isolates were numbered from M1 to M26. Each isolate was given a code indicating its geographical location and catalogue number. The toxin class (*Cas4* and *Cas5*) was referred to the cassiicolin isoform detected and *Cas0* indicated that no cassiicolin gene was detected in the particular isolates. The genetic group (A4 and B4) was determined from the combined sequences of four loci *i.e.* rDNA ITS, ga4, caa5 and act1 (see *Figure 3*).

They were also purified using PCR Purification Kit (QIAGEN, CA) and cloned into pGEM®-T Easy Vector (Promega, WI). The ligation reaction consisted of 5 µl 2× Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector, 3 µl of purified PCR product and 1 µl of T4 DNA Ligase (3 Weiss units/µl). The ligation reaction was incubated for 16 h at 4ºC. The ligated product was transformed into *Escherichia coli* JM109 competent cells. The positive colonies (white) were selected on LB agar media supplemented with 40 µg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 24 µg/mL of IPTG (isopropyl β-D-1-thiogalactopyranoside) and 100 µg/mL of ampicillin. Colony-lysis PCR was performed to screen for inserts in the white colonies. The clear supernatant from colony lysate was subjected to PCR reaction using the specific primers. The positive clones were then cultured overnight at 37ºC and plasmids purified using QIAprep® Spin Miniprep Kit (QIAGEN, CA). The purified plasmids were sent for sequencing to First Base Laboratories Sdn. Bhd. (Malaysia) using SP6 and T7 primers that flank the insert in the pGEM®-T Easy Vector. The nucleotide sequences were generated by 3730XL Genetic Analyzer (Applied Biosystems, CA).

DNA Sequence Analyses

Sequencing results obtained were viewed and analysed using FinchTV_1_4_0. In order to validate the sequences obtained, the forward and reverse sequences were aligned using ClustalW2 multiple sequence alignment tool available at EMBL-EBI database. Generated output format were checked for base ambiguities and miscalls. Sequences were compared to published sequences using the blastn program from NCBI. The amplified genomic DNA, cDNA (excluding introns) and deduced protein sequences were aligned to check for level of similarity and differences among them. Prediction of signal peptide in the deduced cassiicolin protein sequences was performed using SignalP software as discussed previously[12,16].

Phylogenetic Analysis of Four Loci

The 26 isolates were amplified using four sets of primers specific to the ITS1-5.8S-ITS2 region of ribosomal DNA, the random hyper variable loci *ga4* and *caa5* and the actin locus *act1* as described in Déon *et al*.[12] The primers used in this study are listed below in *Table 2*.

TABLE 2. LIST OF PRIMERS USED IN THIS STUDY

Primers	Sequences	Target gene
F19	CGGGGAGGTATCAGGTGTGAG ATA	Cas
R26	CAGAACAAGCCAAAAGAGAAC TAC	Cas
ITS1	TCCGTAGGTGAACCTGCGG	ribosomal DNA ITS[23]
ITS4	TCCTCCGCTTATTGATATGC	ribosomal DNA ITS[23]
GA4F	CCTGCTCCGACTTTGTTGAG	ga4[1]
GA4R	GTCTGGGAGCAGCAAAGACT	ga4[1]
CAA5F	GTCCACAAGTGGAACCTCGT	caa5[1]
CAA5R	CCTCGTCTGCCAGTTCTTCT	caa5[1]
ACT-512-5	ATGTGCAAGGCCGGTTTCGC	act1[24]
ACT-783-R	TACGAGTCCTTCTGGCCCAT	act1[24]

The PCR products were sequenced by GATC-biotech (Konstanaz, Germany). The sequences were corrected and aligned using Geneious Pro v5.3.6. The data were analysed using PAUP*4.0b10 and MrBayes interfaces of Geneious Pro v5.3.6 for ML (Maximum Likelihood), MP (Maximum Parsimony) and BI (Bayesian Inference) methods. PAUP*4.0b10 was used to evaluate the homogeneity of different data partition subsets with 1000 replicates. No significant difference in the phylogenetic signal of the four loci (P=0.085) was found when all data partitions were compared. HKY was chosen as the model of nucleotide substitution for ML and BI methods. This was determined by the Modeltest 3.6 program in PAUP* interface of Geneious Pro v5.3.6. Robustness of nodes was assessed with 1000 bootstrap (BS) replicates for the ML analysis. Two replicates of Markov Chain Monte Carlo were implemented for Bayesian analysis with four heated chains and trees sampled every 1000th generations for one million and one hundred thousand generations. Heuristic search was used to conduct MP analysis. Bootstrapping with 1000 replicates was used in order to obtain statistical support value. Shimodaira-Hasegawa test implemented in PAUP*4.0b10 was used to test the differences between phylogenetic topologies.

Detached Leaf Assay

Four *C. cassiicola* isolates were selected, *i.e.* two isolates (CSB16 and CKT05F) representing cluster 1, CKT05D representing cluster 2 and CLN23 representing cluster 3. These isolates were tested for pathogenicity on detached leaves of six *Hevea* clones (RRIM 600, RRIM 2005, PB260, RRIM 928, RRIM 3001 and RRIM 2020). The fungal isolates obtained from rubber plantations in Peninsular Malaysia were cultured on potato sucrose agar. Sporulation was induced by incubating the cultures in the dark and later exposing to fluorescent light[2]. The fungal spores were inoculated on detached *Hevea* leaflets of about 30 days after budburst. The inoculated leaflets were floated on water in large petri dishes with the abaxial surface facing upwards. Five leaflets per *Hevea* clone (one leaflet per petri dish) were used for inoculation with 10 µl of *C. cassiicola* spore suspension (*circa* 2000 spores/ml), at ten different locations per leaflet. The inoculated leaflets were then placed under fluorescent light in an air conditioned room (25ºC). Each experiment was subjected to three repetitions.

The extent of fungal infection was assessed six days after inoculation essentially as described elsewhere[4]. An infection was considered to have occurred when the leaf tissues at the site of inoculation turned dark. The degree of infection was scored as: 0, no visible lesion; 1, visible dark dots under droplets; 2, small dark discolouration below droplet; 3, prominent large lesion without mycelium; 4, prominent larger lesion with infected vein but without mycelium and 5, prominent larger lesion with infected vein and mycelium. *Hevea* clones with score between 0 to 1.99, 2 to 2.99 and 3 to 5 were considered as highly resistant (light infection), resistant (moderate infection) and susceptible (severe infection), respectively. Two way ANOVA was performed with *Hevea* clone and fungal isolate as source of variation. Tukey's Honest Significant Differences (HSD) test was carried out to find significant differences between means of severity of infection among the test clones and isolates.

RESULTS

Genomic PCR was performed on 26 *C. cassiicola* isolates (*Table 1*) using the gene specific primers F19 and R26 framing the cassiicolin gene from the Malaysian isolate CSB6 (GenBank Accession No. JF915178)[12]. These primers are able to amplify sequences

from the *Cas3*, *Cas4* or *Cas5* cassicolin genes (data not shown). In total, 13 isolates were found to harbour a cassiicolin gene under our PCR conditions. Other primer couples targeting the *Cas1*, *Cas2* and *Cas6* genes failed to amplify any sequence (data not shown). However this does not rule out the possibility of the presence of other cassiicolin genes with a greater degree of nucleotide differences that remain unamplified with these primers.

The deduced protein sequences showed that the cassiicolin gene detected in isolate CKT05D encodes the *Cas4* isoform, while the 12 other isolates harbour a gene encoding the *Cas5* isoform as previously described by Déon and co-workers[12,16]. No *Cas* gene (*Cas0*) was detected in the remaining 13 isolates. The 13 cassiicolin gene sequences were deposited in the database (GenBank Accession Nos. KM873315 – KM873327).

Multiple sequence alignment performed on the 12 genomic sequences encoding *Cas5* revealed no difference among them. They were also fully identical to the previously described *Cas5* genes (GenBank Accession Nos. JF915173 to JF915180)[12]. The *Cas4* gene from isolate CKT05D was fully identical to the previously described *Cas4* genes (GenBank Accession Nos. JF915171 and JF915172)[16]. The alignment of the *Cas4* genomic sequence (from isolate CKT05D) with a representative of the *Cas5* genes is shown in *Figure 1.1*. Three exons and two introns were detected and their positions were conserved in all the 13 cassiicolin genomic sequences. Another multiple sequence alignment was performed using cDNA representatives of *Cas4* and *Cas5* gene (*Figure 1.2*). Nucleotide differences were detected in several locations, including seven within the exon sequences. Alignment of the deduced amino acid sequence of both representatives is depicted in *Figure 1.3*. A change at position 16 of the amino acid sequence was detected in CKT05D where Threonine (T) was substituted for Alanine (A) in the other isolates. Another noteworthy difference at nucleotide level is that the stop codon of CKT05D is TAG whereas it is TAA in the other isolates. However, no difference was detected in the mature cassiicolin protein sequence of all 13 isolates tested in this study (*Figure 1.3*). Toxin classes *Cas0*, *Cas4* and *Cas5* were assigned to the test isolates based on the classification by Deon *et al.*[12,15,16].

A phylogenetic analysis was conducted on the 26 *C. cassiicola* isolates using the combined sequences of four loci (rDNA ITS, ga4, caa5 and act1), as described previously[1,12]. Phylogenetic trees were built using Maximum Likehood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI), but only the tree obtained with the ML method is presented (*Figure 2*). Three clusters were obtained in rather good concordance with the toxin classes. The first cluster consists of isolates (C68-6, C68-8, CSB3, CSB5, CSB6, CSB11, CSB16, CKT1, CSB05A, CSB05B, CKT05C and CKT05F) that carry a *Cas5* gene and were thus classified as toxin class *Cas5* and two isolates (CSD1 and CBPP2) which were classified as *Cas0*. The second group that segregates from cluster 1 contains a single isolate (CKT05D) carrying a *Cas4* gene (toxin class *Cas4*). The third group consists of the remaining 11 isolates, classified as *Cas0* (no detectable *Cas* gene): CLN4, CLN9, CLN11, CLN16, CLN23, CTD1, CTD2, CBS6, CSB15, CBN5 and CLKK1.

The nucleotide sequences of the four loci were registered under the accession numbers: KF810854 - KF810919 and JF915169 - JF915172 (rDNA ITS), KF810784 - KF810853 (ga4), KF810714 - KF810783 (caa5) and KF810644 - KF810713 (act1).

A second phylogenetic analysis using the same combined loci was conducted from a large set of *C. cassiicola* isolates comprising

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1_Cas4    AAATCTTCTAAACATTGTCGATTCCAGATCTTTCAAACCTGTCCCATACATTTGCTACAT 60
12_Cas5   AAATCTTCTAAACATTGTCGATTCCAGATCTTTTAAACCTGTCCCATACATTTGCTATAT 60
          ********************************** ********************** **

1_Cas4    AATGAAATACCTCCCTATCCTCATCTCGGCTTTCGTAGCAGCCGTTACTGCGGCTCCACA 120
12_Cas5   AATGAAATACCTCCCTATCCTCATCTCGGCTTTCGTAGCAGCCGTTGCTGCAGCTCCACA 120
          ********************************************** **** ********

1_Cas4    AGATCCGTCTGCTGTGGCACCTTTGCTCCCTAGACAGTCTTGCGTACGTCTTGACTCATC 180
12_Cas5   AGATCCGTCTGCTGTGGCACCTTTACTCCCTAGACAGTCTTGCGTACGTCTTGACTCATC 180
          ************************ ************************************

1_Cas4    GAATGACAGCCAGGAAATGAAACAACATGTAGCTAATAGTAGATAAAGGTATCGTGTGTC 240
12_Cas5   GAATGACAGCCAGGAGATGAAACAACATGTAGCTAATAATAGATAAAGGTATCATGTGTC 240
          *************** ********************** ************** ******

1_Cas4    AATTTTGGCAATGGGTTCTGTGGCGATAACTGTGGCAATTCCTGGGCTGTAAGTATTTAA 300
12_Cas5   AATTTCGGCAATGGGTTCTGTGGCGACAACTGTGGCAATTCCTGGGCTGTAAGTATTTAA 300
          ***** ******************** ********************************

1_Cas4    CATCTGTTTCTCAAATCTAAAGCTAATTTGCATACAGTGTTCGGGCTGTTAGCTTAAGTA 360
12_Cas5   TATCTGTTTCTCAAATCTAAAGCTAATGTGCATACAGTGTTCGGGCTGTTAACTTAAGTA 360
           ************************** ********************** ********

1_Cas4    GCATTCTGGAGAATTGTGGTCCATAAGACAGTCTAGCTTGAATACTATAGCTAGCTGTTG 420
12_Cas5   GCATTCTAGAGAATTATGGTCCACAAGACAGTCTAGCTTGAACACTATAGCTAGCTGTTG 420
          ******* ******* ******* ****************** ******************

1_Cas4    CTACAAGTCTACAGGGTGGCAGTACTGATTGCCTATATAGGCAAATTTGTTGCTACCAAG 480
12_Cas5   CCACAAGTCTACAGGGTTGCAGTACTGATTGCCTATATAGGCAAACCTGTTGCTACCAAG 480
          * *************** ***************************  *************

1_Cas4    TATTCTGGACTGTTTCTCTCGTTACTTGTATATACTTGGCGCTATATATACACCACACTC 540
12_Cas5   TATTCTGGACTGTTTTTCTTGTTACTTATATACAATTGGCGCTATATATACACCACACTC 540
          *************** *** ******* **** * *************************

1_Cas4    ATTTACATACCGCAAATCCAAATCCTTGCAGATCC 575
12_Cas5   ATTTACATACCGCAAATCCAAATCCTTGCAGATCC 575
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Figure 1.1 Genomic sequence alignment of the cassiicolin genes Cas4 *(1 isolate) and* Cas5 *(12 isolates). The* Cas5 *genes were identical in all 12 isolates. The three exon regions are marked in bold. The conserved splice donor (GT) and splice acceptor (AG) are highlighted. The Start (ATG) and Stop (TAA or TAG) codons are underlined.*

the 26 Malaysian isolates of this study together with 127 isolates from various hosts and geographical origins as in the study by Déon *et al*.[12] (*Figure 3*). The objective was to place the 26 Malaysian isolates in the previously described phylogenetic tree which covers the largest diversity of *C. cassicola* isolates so far in terms of hosts and geographical origins. All isolates from the same cluster in *Figure 2* were grouped in a single clade in *Figure 3*: *Cas5* isolates together with the *Cas0* isolates CSD1 (M19) and CBPP2 (M20) were grouped in Clade B4; *Cas0* isolates of cluster 3 in *Figure 2* adhered to clade A4 in *Figure 3*. However, CKT05D which carried the *Cas4* gene was not highly supported in any clade.

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1_Cas4    ATGAAATACCTCCCTATCCTCATCTCGGCTTTCGTAGCAGCCGTTACTGCGGCTCCACAA 60
12_Cas5   ATGAAATACCTCCCTATCCTCATCTCGGCTTTCGTAGCAGCCGTTGCTGCAGCTCCACAA 60
          ********************************************* **** *********

1_Cas4    GATCCGTCTGCTGTGGCACCTTTGCTCCCTAGACAGTCTTGCGTATCGTGTGTCAATTTT 120
12_Cas5   GATCCGTCTGCTGTGGCACCTTTACTCCCTAGACAGTCTTGCGTATCATGTGTCAATTTC 120
          ********************** ********************** ***********

1_Cas4    GGCAATGGGTTCTGTGGCGATAACTGTGGCAATTCCTGGGCTTGTTCGGGCTGTTAG 177
12_Cas5   GGCAATGGGTTCTGTGGCGACAACTGTGGCAATTCCTGGGCTTGTTCGGGCTGTTAA 177
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Figure 1.2. Multiple sequence alignment of cassiicolin cDNA sequence of Cas4 *(1 isolate) and* Cas5 *(12 isolates). The Start (ATG) and Stop (TAA or TAG) codons are underlined.*

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1_Cas4     MKYLPILISAFVAAVTAAPQDPSAVAPLLPRQSCVSCVNFGNGFCGDNCGNSWACSGC 58
12_Cas5    MKYLPILISAFVAAVAAAPQDPSAVAPLLPRQSCVSCVNFGNGFCGDNCGNSWACSGC 58
           ***************:*****************************************
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Figure 1.3. Multiple sequence alignment of Cas4 *(1 isolate) and* Cas5 *(12 isolates) cassiicolin deduced amino acid sequences. The mature cassiicolin region is underlined. The signal peptide is marked in bold.*

Detached leaf assays were performed with representatives of the three groups of *C. cassiicola* isolates on *Hevea* clones RRIM 600, RRIM 2005, PB260, RRIM 928, RRIM 3001 and RRIM 2020, as shown in *Figure 4*. The sole representative of cluster 2 - CKT05D was the most aggressive isolate, causing severe infection on all the tested clones except on RRIM 3001 and RRIM 2020, with a maximum on RRIM 600. CKT05F from cluster 1 was generally the least aggressive, causing a mild necrotic lesion across the board. CSB16 (cluster 1) and CLN23 (cluster 3) on the other hand showed moderate to low levels of infectivity, although with different profiles among the various test clones. With CLN23, symptom intensity was the highest on PB260 and the lowest on RRIM 600. On the other hand, with CSB16, the highest symptom intensity was observed on RRIM 600 while the lower intensities were observed on RRIM 2020 and PB260.

The ANOVA shown in *Table 3* indicates that the mean of severity of infection was significantly influenced by the interaction between test *Hevea* clone and fungal isolate at the alpha of 0.05. The variance ratio (VR) obtained is 3.52 ($F(15, 96) = 3.52$, $p < 0.05$). However, the effect of fungal isolate is of higher significance compared to the mean of severity of infection where the VR is 14.74.

Generally, the infection ranged from light to moderate with the lowest score recorded on RRIM 3001 (Mean=1.280, SD=0.53), while the highest was observed on RRIM 928 (Mean= 3.285, SD=1.44) (*Table 4*).

As shown in *Table 5*, CKT05D was the most aggressive (Mean=3.203, SD=1.73), while CKT05F was the least aggressive isolate (Mean=1.480, SD=0.73). As for isolates CSB16 and CLN23, the virulence was significantly similar with mean of score between 2.227 to 2.383.

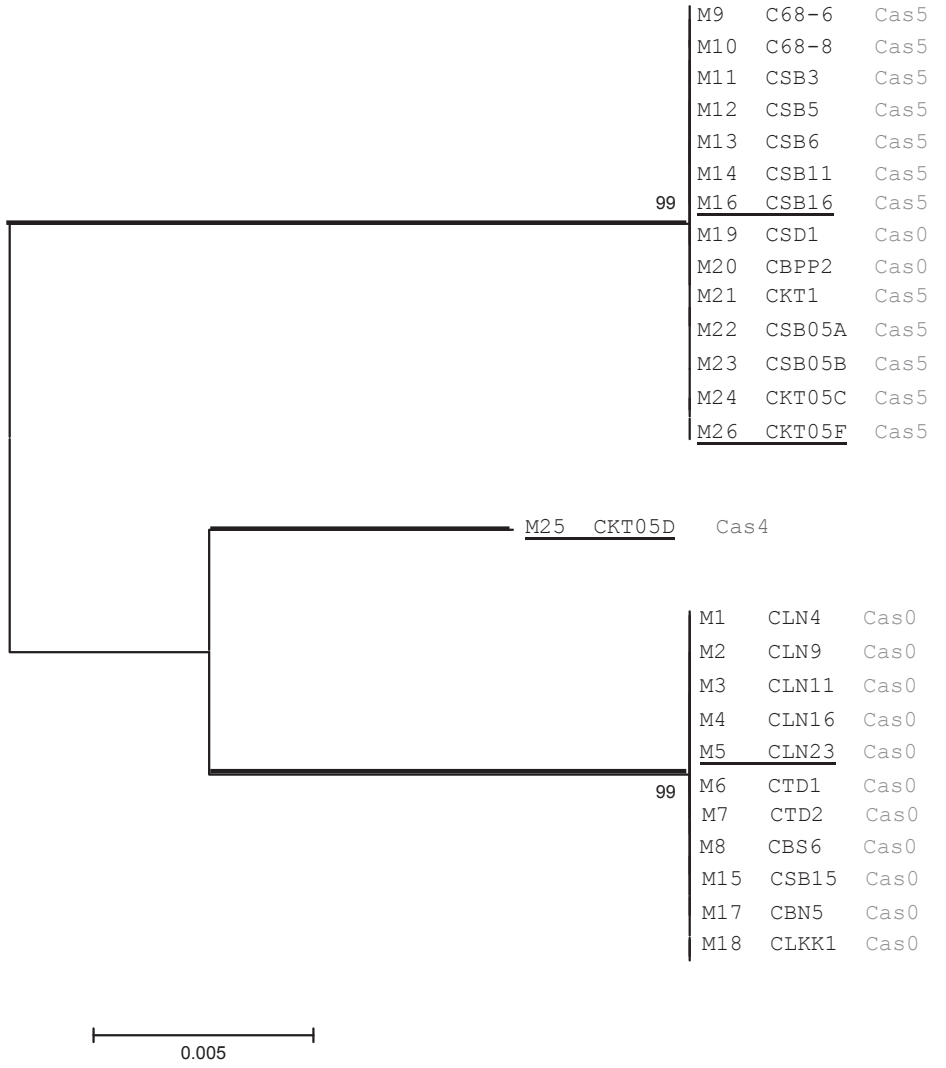


Figure 2. Phylogenic tree of the 26 Corynespora cassiicola *isolates from rubber plantations in Malaysia. The tree was generated from the combined sequences of four loci* i.e. *rDNA ITS, ga4, caa5 and act1 using the ML (Maximum Likelihood) method, with BS (Bootstrap) values indicated at nodes. The toxin class of each isolate (*Cas0*,* Cas4 *or* Cas5*) is indicated adjacent to the isolate name. The isolates used in the detached leaf assay are underlined.*

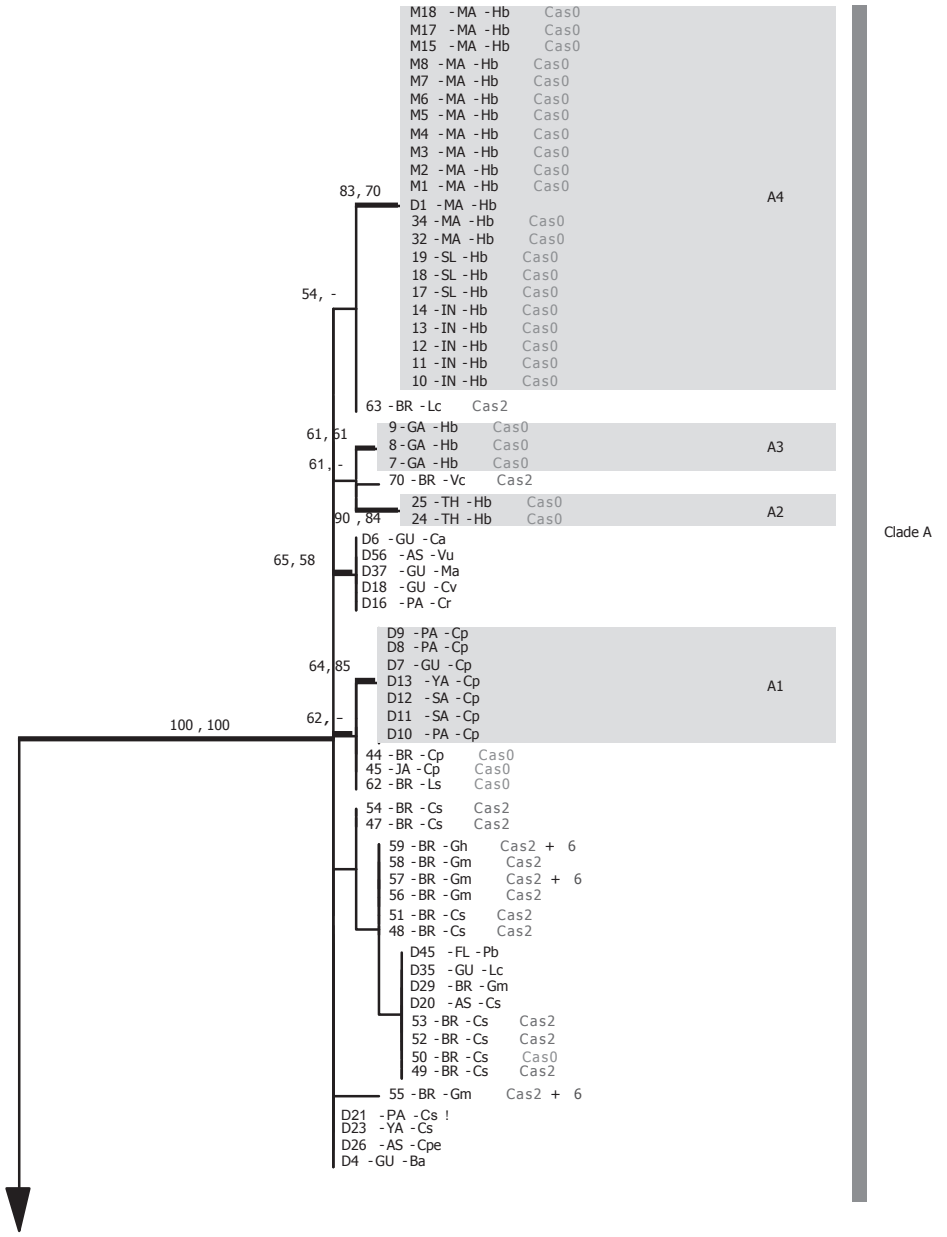


Figure 3. Integration of 26 Corynespora cassiicola *isolates from Malaysia (M1 to M26) in the general phylogenetic tree of the fungal isolates obtained from different geographic origins and hosts (Déon* et al.[12]*). The tree was generated from the combined sequences of four loci* i.e. *rDNA ITS, ga4, caa5 and act1, using the ML (Maximum Likelihood) method. Bootstrap values derived from the ML and MP (Maximum Parsimony) analysis are indicated successive (ML, MP), at nodes. PB (Posterior Probabilities) (>94) from the BI (Bayesian Inference) method are indicated as thick branches. The toxin class of each isolate is indicated adjacent to the isolate name.*

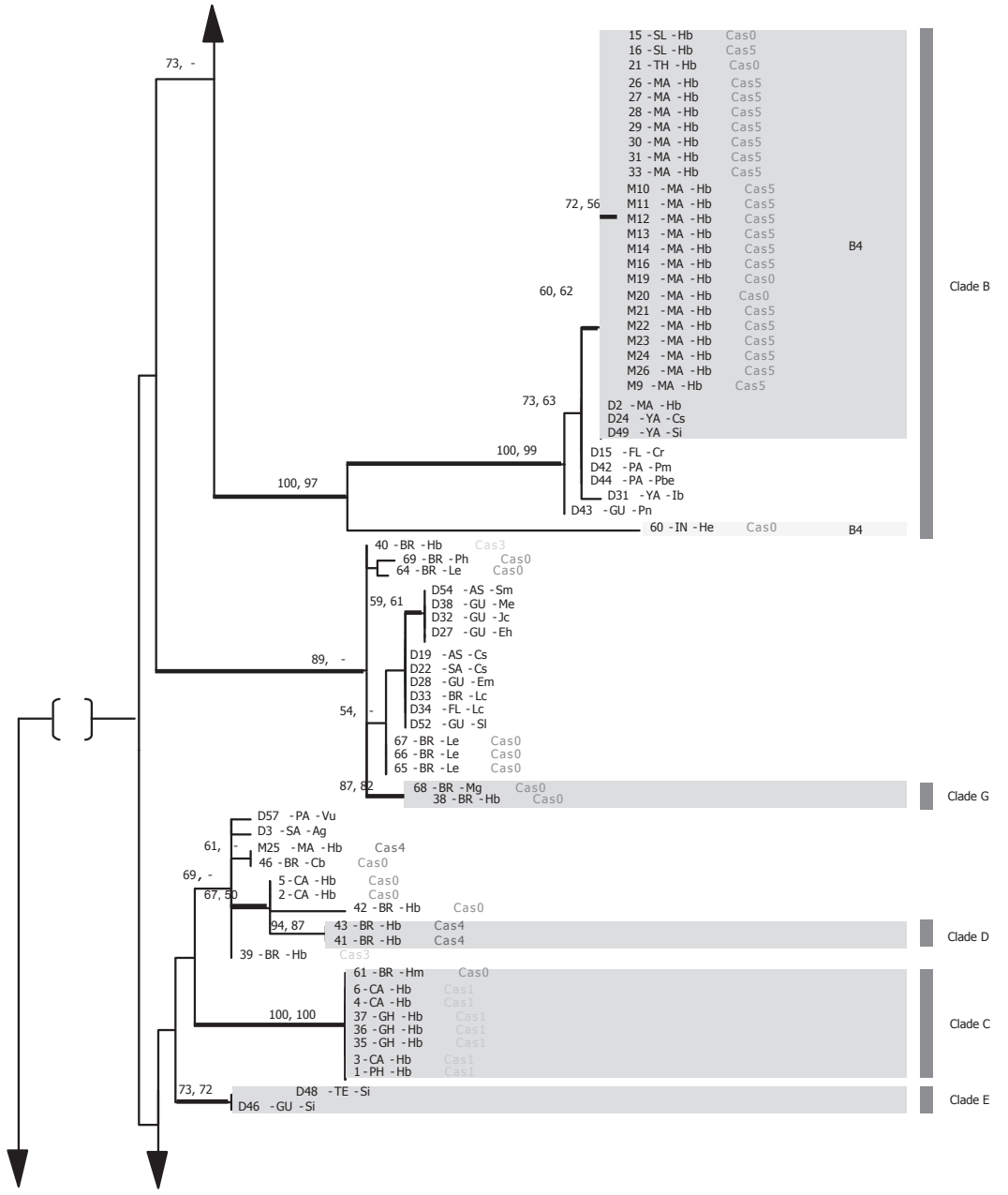
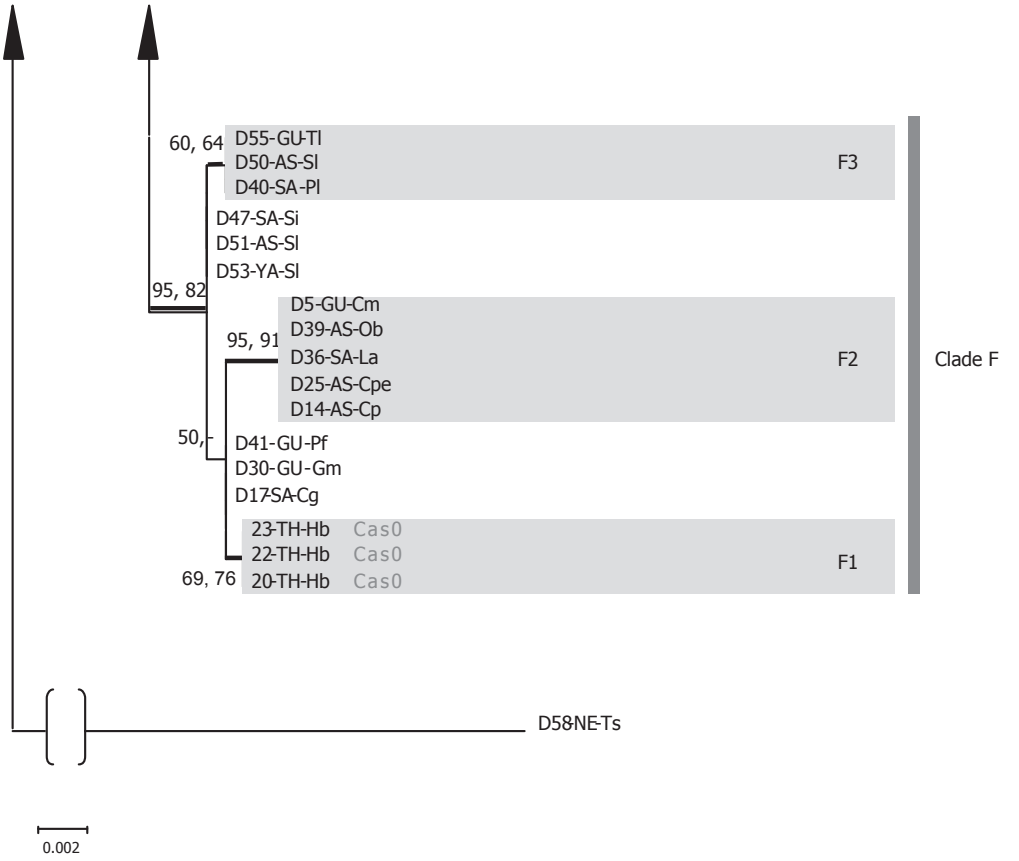


Figure 3 (cont.). Integration of 26 Corynespora cassiicola *isolates from Malaysia (M1 to M26) in the general phylogenetic tree of the fungal isolates obtained from different geographic origins and hosts (Déon* et al.[12]*). The tree was generated from the combined sequences of four loci* i.e. *rDNA ITS, ga4, caa5 and act1, using the ML (Maximum Likelihood) method. Bootstrap values derived from the ML and MP (Maximum Parsimony) analysis are indicated successive (ML, MP), at nodes. PB (Posterior Probabilities) (>94) from the BI (Bayesian Inference) method are indicated as thick branches. The toxin class of each isolate is indicated adjacent to the isolate name.*



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A significant statistical interaction indicated that the severity of infection among the fungal isolates depends on the test *Hevea* clones ($F_{15,96}$ = 3.52, p < 0.05). The nature of this interaction is illustrated in *Figure 5*. Simple effect analyses were used to further examine the interaction between isolates and clones. These analyses indicated that the test fungal isolates did have a statistical effect on the severity of infection in clone RRIM 600 ($F_{3, 96}$ = 11.432, P < 0.05), PB260 ($F_{3,96}$ = 10.409, P < 0.05) and RRIM 928 ($F_{3,96}$ = 6.754, P < 0.05). However, the fungal isolates had no influence on the severity of infection when tested with clone RRIM 2005, RRIM 2020 and RRIM 3001 (*Table 6*).

TABLE 3. TWO WAY ANOVA WITH *HEVEA* CLONE AND *CORYNESPORA CASSIICOLA* ISOLATE AS SOURCE OF VARIATION

Source of variation	df	MS	VR
Clone	5	14.00	13.77***
Isolate	3	15.00	14.74***
Clone x Isolate	15	3.58	3.52***
Error	96	1.02	

ns (not significant), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), df = degree of freedom, MS = mean square, VR = variance ratio

TABLE 4. COMPARISON OF MEAN OF SCORE AMONG DIFFERENT *HEVEA* CLONES

Clone	Mean of score
RRIM 600	2.605^{bc}
RRIM 928	3.285^{c}
RRIM 2005	3.055^{bc}
RRIM 2020	1.390^{a}
RRIM 3001	1.280^{a}
PB260	2.325^{b}

Means with the same letter are not significantly different at $P < 0.05$ based on Tukey $df_E = 20$, MSE = 1.016

TABLE 5. COMPARISON OF MEAN OF SCORE ON DIFFERENT *CORYNESPORA CASSIICOLA* ISOLATES

Isolate	Mean of score
CKT05D	3.203^{c}
CSB16	2.227^{b}
CLN23	2.383^{b}
CKT05F	1.480^{a}

Means with the same letter are not significantly different at $P < 0.05$ based on Tukey $df_E = 30$, $MS_E = 1.016$

TABLE 6. PATHOGENICITY OF *CORYNESPORA CASSIICOLA* ISOLATES ON SELECTED *HEVEA* CLONES

Isolate	Average infection score on different *Hevea* clones					
	RRIM 600	RRIM 928	RRIM 2005	RRIM 2020	RRIM 3001	PB260
CKT05D	4.80^{b}	4.32^{b}	3.88^{a}	1.64^{a}	1.44^{a}	3.14^{ab}
CKT05F	1.46^{a}	1.96^{a}	2.42^{a}	1.00^{a}	1.00^{a}	1.04^{a}
CSB16	2.46^{a}	4.22^{b}	3.40^{a}	1.12^{a}	1.00^{a}	1.16^{a}
CLN23	1.70^{a}	$2.64a^{b}$	2.52^{a}	1.80^{a}	1.68^{a}	1.68^{a}

Means with the same letter are not significantly different at $P < 0.05$

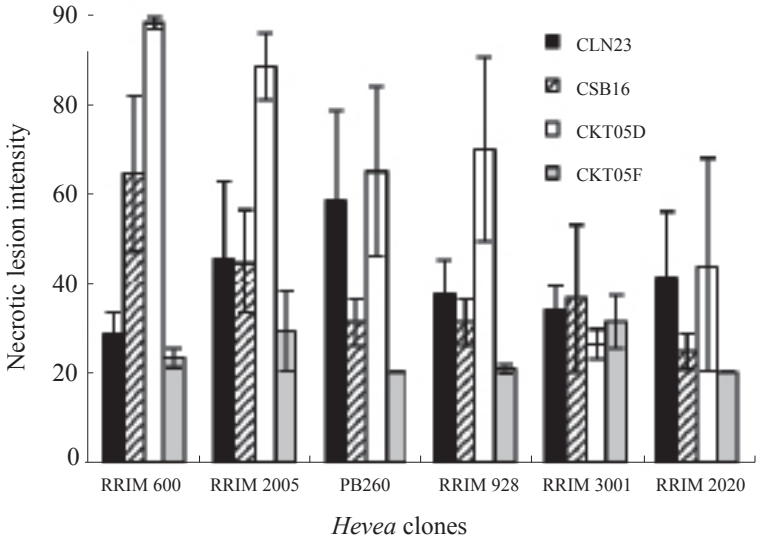

Figure 4. Pathogenicity profiles of four Corynespora cassiicola *isolates representing three clusters on six* Hevea *clones. Interaction plot showing mean intensity of necrotic lesions formed at the site of treatment in the detached leaf assay on RRIM 600, RRIM 2005, PB260, RRIM 928, RRIM 3001 and RRIM 2020. Treatments: CLN23 filled box, CSB16 hatched box, CKT05D open box, and CKT05F grey box.*

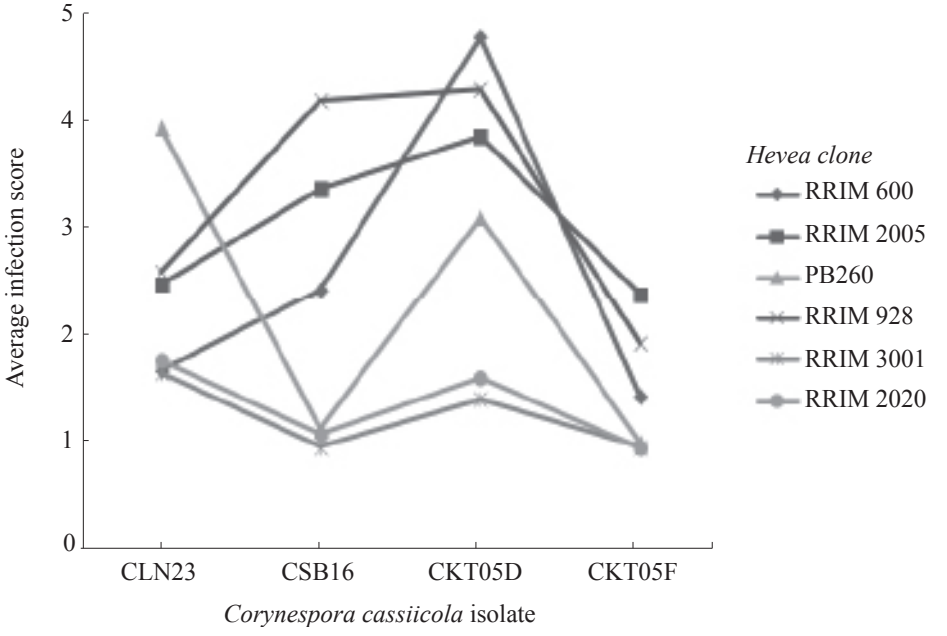

Figure 5. Interaction plot between the test Corynespora cassiicola *isolates and* Hevea *clones showing the severity of infection between two factors interaction is statistically significant.*

DISCUSSION

The gene specific PCR approach employed in this study revealed the presence of a cassiicolin gene in 13 *C. cassiicola* isolates collected from rubber plantations throughout Malaysia, with two possible forms (*Cas4* or *Cas5*). Another group of 13 isolates showed no detectable Cas gene (toxin class *Cas0*). To date, six cassiicolin gene variants named *Cas1* to *Cas6* have been identified[12]. Some isolates from Malaysia were previously shown to carry the *Cas5* gene, while others were *Cas0* but this is the first report on a *Cas4* gene found in isolates from Malaysia.

From the phylogenetic analysis based on four combined loci (*Figures 2* and *3*), it can be concluded that, the Malaysian isolates were divided in two big clusters and one single isolate, in rather good concordance with the toxin class. The first cluster (*Figure 2*) is comprised of all isolates carrying the *Cas5* gene as well as two isolates in which no *Cas* gene could be PCR-detected in our conditions. In the global phylogenetic analysis (*Figure 3*), this cluster was fully integrated in Clade B4, previously shown to contain all the so far identified *Cas5* isolates[12]. Isolate CKT05D carrying the *Cas4* gene was singled out in cluster 2 (*Figure 2*). In the global phylogenetic analysis (*Figure 3*), it did not fall in any highly supported clade although it was in near distance with two *Cas4* isolates from Brazil grouped under Clade D[12]. The third cluster only contained isolates with no detectable *Cas* gene and was fully integrated into Clade A4 (*Figure 3*), containing *Cas0* isolates only.

In a previous analysis using ISSR markers, a total of 21 local *C. cassiicola* isolates were segregated into two distinct clusters (1 and 2), wherein cluster 1 was further segregated into two sub-clusters (1A and 1B) with 1B harbouring only one isolate, CKT05D[17]. In comparison with the present, there is no disagreement in the segregation pattern of the common isolates that were examined in both studies, including the variation that was discerned with isolate CKT05D.

In order to investigate the relation between the genetic groups and the pathogenicity, a leaf assay was conducted with isolates representative of the three groups on four *Hevea* clones. The test revealed contrasting responses but no clear relation between the genetic group (and toxin class) and the level of virulence. Indeed, two isolates that were representatives of the same genetic group (B4) and toxin class (*Cas5*), *i.e*. CSB16 and CKT05F, showed different levels of virulence, with CSB16 moderately virulent and CKT05F very weakly virulent. Similarly, in a previous study by Déon *et al*.[12] two Malaysian isolates of the same B4/*Cas5* type were found moderately and weakly virulent, respectively. A difference in *Cas5* gene expression level may explain such discrepancies of the symptoms, while the role of other yet to be determined effector(s) is not ruled out. In the same study, a Brazilian isolate carrying the *Cas4* gene was found virulent with medium aggressiveness on clone RRIM 600, like CKT05D (also carrying *Cas4*) in the present study.

The leaf assay also revealed a clone/isolate interaction: the isolates CKT05D from cluster 2 (classified as -/*Cas4*) and CSB16 from cluster 1, (classified as B4/*Cas5*) showed similar pathogenicity profiles, with higher symptom intensity on RRIM 600 compared to PB260. On the contrary, CLN23 from cluster 3 (classified as A4/*Cas0*) differed, with higher symptom intensity on PB260 compared to RRIM 600. Whether the *Cas* genes are expressed and involved in this clone/isolate interaction remains to be demonstrated.

The fact that CLN23, deprived of detectable *Cas* gene, was nevertheless moderately virulent on clone PB260, suggests the existence of another effector involved in the disease symptoms on this clone. This effector could be another cassiicolin variant or a completely different causal factor. It is important to note that the isolates sampled from newer *Hevea* clones like RRIM 2008, RRIM 2020 and RRIM 2025 were all classified as *Cas0*.

In the past, a number of molecular marker techniques have been used in the detection, differentiation, and classification of *C. cassiicola* isolates[6,7,17–22]. Attempts were also made to draw a correlation between the various groupings obtained and the characteristics of the *C. cassiicola* isolates *e.g*. pathogenicity, geographical region or host plant genotype. While these were conclusive in some cases[6,7,19,20], others did not reveal any clear correlation[21,22]. Interestingly, an investigation using Single Nucleotide Polymorphisms (SNPs) managed to show a reasonable correlation between the pathogenicity profiles and the genetic groups[18]. In another report[17], isolate CKT05D particularly, showed a pathogenicity profile dissimilar to the established pathovars of the *C. cassiicola* isolates from rubber plantations in Malaysia[17]. It appears that the phylogenetic groups B4 and A4 obtained from the present analysis and as reported earlier by Deon *et al*.[12] may correspond to the previously described race 1 and 2 respectively[4]. The results also point to the presence of a third race in Malaysia, represented by isolate CKT05D although it is not phylogenetically supported so far. Further studies are required to investigate the role of the cassiicolin genes (*Cas5* and *Cas4* respectively) in pathogenicity of race 1 and 3 isolates. On the other hand, the detection of *Cas0* indicates that the causal factors of pathogenicity in race 2, isolates remain to be identified.

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