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Intraspecific Variability of Corynespora cassiicola *Inferred from Single Nucleotide Polymorphisms in ITS Region of Ribosomal DNA*

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Two single nucleotide polymorphisms (SNPs) were detected in the internal transcribed spacer region of the nuclear ribosomal DNA (rDNA-ITS) of 21 Corynespora cassiicola *isolates obtained from a number of* Hevea *clones grown in rubber plantations in Malaysia. The two SNPs correlated with the physiological races of the isolates. Database searches yielded another 28* C. cassiicola *isolates from diverse hosts and geographical regions. With this inclusion, a total of seven SNPs and two indels in the rDNA-ITS region were detected from all 49* C. cassiicola *isolates. The knowledge of intraspecific variability (SNPs and indels) could prove useful in the delineation of physiological races or pathotypes of this fungus.*

Keywords: *Corynespora cassiicola*, *Hevea brasiliensis*, ITS rDNA region, Single Nucleotide Polymorphisms

Abbreviations: BLAST (Basic local alignment search tool); ITS rDNA (Internal Transcribed Spacer region of the Ribosomal DNA); SNPs (Single Nucleotide Polymorphisms)

Footnotes: Nucleotide sequence data reported are available in the GenBank databases under the accession number(s) from EU364535 to EU364555.
Single nucleotide polymorphisms data reported are available in the dbSNP database (Build 130) under the submitter SNP (ss) accession numbers from ss105106974 to ss105106982 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewBatch.cgi?pbid=60542&pid=10196&phan=PLMOLBIOLGRPUPM&lpid=EastAsia_isolates).

Corynespora cassiicola is a widespread plant pathogenic fungus; it has been recorded in over 70 countries and from more than 300 plant species including fruits, vegetables, grains, perennial crops, forestry and various ornamental plants[1]. Strains or races of *C. cassiicola* from different hosts have been reported but are not always cross infective to

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other hosts[2–4]. In rubber (*Hevea brasiliensis*) plantations, this pathogen causes *Corynespora* leaf fall disease (CLFD), one of the most important diseases in many rubber producing countries[5]. Different strains or races of *C. cassiicola* that infect rubber have also been identified and genetic relationships among isolates have been established[6–10]. In Malaysian rubber plantations, two races of the pathogen are known to exist; Race 1 isolates severely infect the earlier clones (*e.g*. RRIM 600, GT1 and IAN 873) and have milder infection on the newer clones (*e.g*. RRIM 2020 and PB 260); while Race 2 isolates severely infect these newer clones[6]. In our previous study[10], Inter Simple Sequence Repeat (ISSR) polymorphism and detached leaf assay were employed to analyse 21 *C. cassiicola* isolates obtained from a number of *Hevea* clones grown in rubber plantations from several states in Malaysia. Therein, eleven isolates collected from the states of Johore and Selangor were grouped into Race 1, while nine isolates collected from other states were grouped into Race 2 and a unique isolate (CKT05D) collected from Johore showed pathogenesis dissimilar to either races. In the present study, the internal transcribed spacer region of the nuclear ribosomal DNA (ITS rDNA) sequences were used to analyse the intraspecific variability of these 21 isolates and another 28 isolates from diverse hosts in several other countries that were retrieved from the GenBank database[11], and here we report the existence of Single Nucleotide Polymorphisms (SNPs) in this region of rDNA.

MATERIALS AND METHODS

Twenty one isolates of *C. cassiicola* collected from 1998 to 2005 on different rubber clones grown in several states of Malaysia as described in our previous publication[10] were used for the analysis (*Table 1*). The procedures to harvest mycelia and extract genomic DNA have also been reported previously[10]. The ITS rDNA of 21 isolates from Malaysia was amplified using universal primers ITS1 and ITS4[12]. The primers were synthesised at First BASE Laboratories Sdn Bhd, Malaysia. PCR amplification was performed in a 25 µL reaction containing 1 × PCR buffer (Finnzymes, Finland), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of ITS1 primer, 1 µM of ITS4 primer, 0.5 U of DyNAzyme™II DNA Polymerase (Finnzymes, Finland) and 5 ng of template DNA. The amplification was conducted in a thermocycler (DNA Engine® Peltier Thermal Cycler PTC-200, MJ Research, USA) with initial denaturation at 94ºC for 2 min, followed by 35 cycles at denaturing temperature 94ºC for 20 s, annealing temperature 53ºC for 30 s, extension temperature 72ºC for 30 s and the final extension at 72ºC for 5 min. All PCR reactions were performed in three technical replications. The presence of amplified DNA fragment was confirmed using agarose gel electrophoresis. A quantity of 5 µl reaction solution was run on 1% agarose gel using 1 × TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) at 70 V for 45 min at room temperature. The gel was stained with ethidium bromide, visualised under UV light and photographed using a gel documentation system (GeneSnap, Ver 6.03, Syngene Laboratories, USA). The size of amplified DNA fragment was estimated by comparison with a 2-Log DNA Ladder (0.1-10 kb) marker (BioLabs, USA). The PCR products from three replications of each isolate were accumulated and purified using QIAquick PCR purification kit (CAT No. 28104, Qiagen, Germany). The purified PCR products were sequenced by a commercial sequencing service (First BASE Laboratories Sdn. Bhd., Malaysia) using the primers ITS1 and ITS4[12] with BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems), which is based on Sanger's dideoxy sequencing technology. The sequence

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data of each isolate were refined using BioEdit software (version 7.0.9.0)[13] in which the sequence obtained from reverse primer ITS4 was transformed to reverse complement and aligned with the sequence obtained from forward primer ITS1 for confirming and correcting the ambiguous nucleotides on analysed chromatogram. The refined sequences of 21 isolates were then aligned using the Clustal W Multiple alignment (version 1.4)[14] bundled in BioEdit software to assess the nucleotide similarity among isolates. BLAST alignment (BLASTN 2.2.17)[15] was conducted to identify/confirm the species identity of the 21 isolates by analysing the similarity of these sequences with the sequences of the same species available in GenBank[11]. These 21 ITS rDNA sequence data were then submitted to GenBank; their accession numbers ranged from EU364535 to EU364555 (*Table 1*). To detect the SNPs among *C. cassiicola* isolates, a total of 49 nucleotides sequences including the 21 sequences introduced in this study and those of 28 *C. cassiicola* isolates deposited in the GenBank database[11] (*Table 2*) were aligned using the Clustal W Multiple alignment (version 1.4)[14] bundled in the BioEdit software.

TABLE 1. SOURCES, RACES AND GENBANK ACCESSION NUMBERS OF *CORYNESPORA CASSIICOLA* ISOLATES FROM MALAYSIA

No	Isolate	State of origin	Race	GenBank Accession Number
1	CKT1	Johore	1	EU364535
2	CKT05A	Johore	1	EU364536
3	CKT05B	Johore	1	EU364537
4	CKT05C	Johore	1	EU364538
5	CKT05D	Johore	Unknown	EU364539
6	CKT05E	Johore	1	EU364540
7	CKT05F	Johore	1	EU364541
8	CSB5	Selangor	1	EU364542
9	CSB6	Selangor	1	EU364543
10	CSB16	Selangor	1	EU364544
11	CSB05A	Selangor	1	EU364545
12	CSB05B	Selangor	1	EU364546
13	CKS1	Perak	2	EU364547
14	CLKK1	Perak	2	EU364548
15	CLN16	Pahang	2	EU364549
16	CLN23	Pahang	2	EU364550
17	CKK1	Kedah	2	EU364551
18	CTD1	Terengganu	2	EU364552
19	CTD2	Terengganu	2	EU364553
20	CBN5	Terengganu	2	EU364554
21	CBS1	Sarawak	2	EU364555

TABLE 2. SOURCES AND GENBANK ACCESSION NUMBERS OF *CORYNESPORA CASSIICOLA* ISOLATES FROM VARIOUS HOSTS IN SEVERAL COUNTRIES

No	Host name	Country of origin	GenBank Accession Number
1	*Heminthosporium complex*	China	AF163087
2	*Camptotheca accuminata*	China	DQ272496
3	*Hevea brasiliensis*	China	EF198115
4	*Cucumis sativus*	China	EF198116
5	*Cucumis sativus*	China	EF198117
6	*Hevea brasiliensis*	China	EU131374
7	*Carica papaya*	China	EU131375
8	*Hevea brasiliensis*	China	EU131376
9	*Hevea brasiliensis*	China	EU196038
10	*Salvia splendens*	China	EU240457
11	*Salvia splendens*	China	EU240458
12	*Salvia splendens*	China	EU240459
13	*Carica papaya*	China	EU735060
14	*Carica papaya*	China	EU735061
15	*Carica papaya*	China	EU735062
16	*Solanum melongena*	China	EU735063
17	*Carica papaya*	China	EU735064
18	*Vigna sp.*	China	EU735065
19	*Sesamum indicum*	China	EU735066
20	*Hevea brasiliensis*	India	EF471932
21	*Capsicum annuum*	Japan	AB366649
22	*Capsicum annuum*	Japan	AB366650
23	*Capsicum annuum*	Japan	AB366651
24	*Capsicum annuum*	Japan	AB366652
25	*Mandevilla amoena*	Japan	AB433532
26	*Cucumis sativus*	Korea	AY238605
27	*Cucumis sativus*	Korea	AY238606
28	*Capsicum annuum*	Korea	EF490450

(GenBank, Benson *et al.*, 2008)

RESULTS AND DISCUSSION

PCR amplifications of total genomic DNA using the primer pair ITS1–ITS4 produced a single PCR product which included ITS1-5.8S rDNA-ITS2 region in all studied isolates (*Figure 1*). DNA sequencing confirmed that the DNA fragments generated from all isolates were equal in length (559 bp). Sequence alignment revealed that the rDNA-ITS regions of all 21 isolates were identical with the exception of two SNPs detected in the ITS1 region. (*Figure 2*). Interestingly, these SNPs correlated with the races of

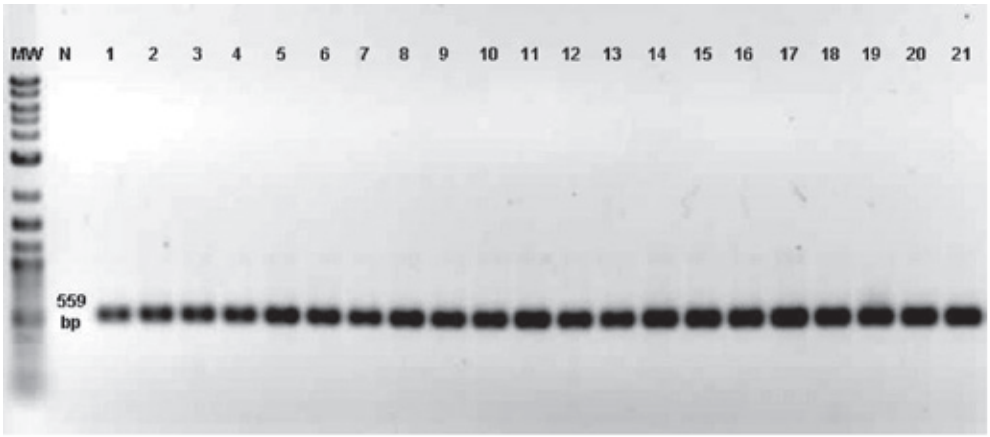

Figure 1. Gel electrophoresis of amplification products obtained from 21 Corynespora cassiicola *isolates using ITS1 and ITS4 primers. Lane 1 (MW) is 2-Log DNA Ladder (BioLabs)*

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                          80        90        100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1_CKT1              TATGAGCACCTCTCGTTTCCTCGGCAGGCTCGCCTGCCAACGGGGACCCACCACAAACCCATTGCAGTAC
2_CKT05A            ...................................................................
3_CKT05B            ...................................................................
4_CKT05C            ...................................................................
5_CKT05D            ..........................C........................................
6_CKT05E            ...................................................................
7_CKT05F            ...................................................................
8_CSB5              ...................................................................
9_CSB6              ...................................................................
10_CSB16            ...................................................................
11_CSB05A           ...................................................................
12_CSB05B           ...................................................................
13_CKS1             ...................................................................
14_CLKK1            ...................................................................
15_CLN16            ...................................................................
16_CLN23            ...................................................................
17_CKK1             ...................................................................
18_CTD1             ...................................................................
19_CTD2             ...................................................................
20_CBN5             ...................................................................
21_CBS1             ...................................................................
C1. Cons.           *************************** ********************************* *****
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Figure 2. Single nucleotide polymorphisms observed at two nucleotide positions (100 and 135) in the ITS1 region of 21 Corynespora cassiicola *isolates. Isolates 1 to 12 were from Johore and Selangor, while isolates 13 to 21 were from other states in Malaysia. The numbering at the top corresponds to the position of the nucleotides counted from the 5' end of the sequence.*

the isolates. Eleven isolates obtained from Johore and Selangor identified as Race 1[10] possessed Cytosine (C) residue while nine other isolates obtained from other states identified as Race 2[10] possessed Thymine (T) at position 135. A unique isolate CKT05D that showed pathogenicity profile distinctively dissimilar from Race 1 and Race 2[10] possessed Cytosine (C) residue instead of Thymine (T) at position 100. This finding further distinguishes CKT05D from the other two races; however, further analyses should be performed to confirm its physiological race and identity.

SNPs are the most common types of mutation that occur in genomic DNA[16–17]. With their abundance and the advancement in the detection technologies for high-throughput genotyping, SNPs are rapidly gaining acceptance as a tool in genetics research[18]. SNPs in the ITS rDNA region of certain fungal species have been reported, *e.g*. *Glomus etunicatum*[19], *Trichophyton tonsurans*[20], and *Agaricus bisporus*[21]. In *C. cassiicola*, seven SNPs and two indels were detected in ITS rDNA region of 49 nucleotides sequences including the 21 sequences introduced in this study and those of 28 *C. cassiicola* isolates obtained from several countries on diverse hosts deposited in the GenBank database; the data were then submitted to the dbSNP database[22]. Description of these SNPs and Indels, and the dbSNP Submitter SNP (ss) accession numbers (from ss105106974 to ss105106982) are shown in *Table 3*. The frequencies of almost all mutant nucleotides were low (varied from 0.01 to 0.11) either existed in only one isolate or a group of isolates associated with a geographical region or host. Thus, it is tempting to speculate that the difference in geographical regions or hosts may have a bearing in the variation or mutation of this fungal species, which may in turn have caused certain mutant nucleotides to occur and hence detected with low level in frequency. However, in the case of the SNP at position 135 observed in this study, the high frequency of T nucleotide (T=0.43) in members from different geographical regions and host has cast doubt on the origin of this anomaly, whether it initially emerged at a specific geographical region and later spread to other regions; further studies are needed to clarify this suggestion.

TABLE 3. DESCRIPTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) AND INDELS DETECTED FROM ITS rDNA REGION OF 49 *CORYNESPORA CASSIICOLA* ISOLATES

No	Local SNP ID	Nulcleotide position	Alternative nucleotide	Nucleotide frequency	ssSNP ID
1	ITS1-nu4	4	C/G	C=0.07/G=0.93	ss105106974
2	ITS1-nu45	45	T/-	T=0.10/-=0.90	ss105106975
3	ITS1-nu100	100	C/T	C=0.04/T=0.96	ss105106976
4	ITS1-nu135	135	T/C	T=0.43/C=0.57	ss105106977
5	5.8S-nu194	194	A/G	A=0.02/G=0.98	ss105106978
6	ITS2-nu391	391	-/G	-=0.02/G=0.98	ss105106979
7	ITS2-nu474	474	A/G	A=0.11/G=0.89	ss105106980
8	ITS2-nu499	499	G/A	G=0.02/A=0.98	ss105106981
9	ITS2-nu550	550	G/A	G=0.03/A=0.97	ss105106982

The ITS rDNA nucleotide sequences of nine isolates identified as Race 2 in Malaysia[10] which contained the nucleotide T at position 135 were identical to eleven deposited sequences obtained from India, China and Korea on several hosts including *H. brasiliensis, Cucumis sativus, Carica papaya, Vigna* sp., *Sesamum indicum, Camptotheca accuminata* and *Capsicum annuum*. This finding implies that the occurrence of Race 2 is widespread in Asia, and that it has a relatively wide host range. Hence, caution should be exercised to ensure that such crops are not utilised in crop rotation or planted as intercrop in rubber plantations where this genotype of *C. cassiicola* has been detected.

CONCLUSION

While the two SNPs from this study correlated with the *C. cassiicola* races that show preference to infect different rubber clones (*H. brasiliensis*) in Malaysia, elsewhere a number of isolates obtained from the same region on the same host also showed identical nucleotide sequences *e.g.* four isolates from Japan on *C. annuum* (AB366649 – 52), and three isolates from China on *Salvia splendens* (EU240457 – 59). A wider study on the variability of ITS rDNA region exposed by SNPs or indels may help to draw correlation with the races or pathotypes of this fungus. If confirmed, SNPs or indels in ITS rDNA region could prove useful as "molecular markers" for identification of the races as well as in disease diagnosis and in diversity analysis of this fungus.

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