



UNIVERSITI PUTRA MALAYSIA

***MORPHOLOGICAL, MOLECULAR CHARACTERIZATION AND
PATHOGENICITY OF COLLETOTRICHUM SPP. ISOLATED FROM LEGUME
CROPS IN PENINSULA MALAYSIA***

FARSHID MAHMUDI

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PATHOGENICITY OF *COLLETOTRICHUM* SPP. ISOLATED FROM
LEGUME CROPS IN PENINSULA MALAYSIA**

By

FARSHID MAHMODI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of
Doctor of Philosophy**

June 2013

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Dedicated to:

All I love,

Specially the soul of my beloved mother in the heaven,

My beloved father,

My beloved wife and my lovely son and daughter “Aydin” and “Rozhan” who have been a constant source of encouragement to continue my study and for thier boundless love, understanding, patience and support throughout my study in Malaysia.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MORPHOLOGICAL, MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF *COLLETOTRICHUM* SPP. ISOLATED FROM LEGUME CROPS IN PENINSULA MALAYSIA

By

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June 2013

Chair: Associate Professor Jugah Kadir, PhD

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The genus *Colletotrichum* is among the most important groups of plant pathogenic fungi worldwide. The ability to accurately diagnose species is vital for the implementation of effective disease control and quarantine measures. Little is known and published about the population, distribution, biology and genetic diversity of *Colletotrichum* species on grain legumes in Malaysia for disease management practices. It is clear that *Colletotrichum truncatum*, *Colletotrichum dematium* and *Colletotrichum gloeosporioides* were the main species responsible for the anthracnose on grain legumes in Malaysia. *Colletotrichum capsici* and *C. truncatum* are unique species, although it has been suggested that *C. capsici* is a synonym of *C. truncatum*. Analysis of molecular data from DNA sequences of multigene loci confirmed the identity and differentiation of these species. The majority species isolated in this

research were in match with the classical morphological descriptions in culture, with the exception of partly overlapping morphological characters. Regardless of host-origination, *Colletotrichum* isolates exhibited different degrees of variability and overlapping in morphological and cultural characteristics. *Colletotrichum truncatum* is known to have a broad host range on the Fabaceae family and other crops such as cabbage (*Brassica chinensis*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*) and lettuce (*Lactuca sativa*). In contrast, the host range of isolates of *C. dematium* and *C. gloeosporioides* is limited to cowpea and soybean, respectively, *Centrosema pubescens* was also a common host. *Colletotrichum truncatum* was reported as more prevalent in soybean than other legumes grown in Malaysia. Pathogenic isolates were genetically indistinguishable from non-pathogenic isolates. Pathogenicity data supported variability in *C. truncatum* structure with diversity in the host. The molecular data showed congruence between genetic diversity and host populations. A clear insight of the genetic diversity and relationship of *Colletotrichum* spp. isolates has now emerged. The data also revealed high genetic diversity dispersed to the smallest scale, at the regional or field level. No apparent geographic effect was observed.

The combined data of six genes comprised of 2771 characters after alignment, of which 985 characters were parsimony informative. Parsimony and neighbor-joining analysis produced similar topology and all *Colletotrichum* isolates were clustered into 3 well-defined clades with high bootstrap support, representing three distinct species. Histone

and GAPDH genes contain the most phylogenetically informative characters among examined taxa, suggesting these loci with high number of polymorphic nucleotide site can be used in phylogenetic studies to build highly resolved phylogenies at the intraspecific level and closely related species. ISSR and RAPD markers used to investigate the genetic composition of *Colletotrichum* species from legumes were able to differentiate the species examined. Molecular polymorphism assessed by RAPD and ISSR revealed a level of molecular variability greater than that characterized using multigene sequence analysis within and among *Colletotrichum* species. However, the results between markers were congruent, but the effective marker ratio and subsequent to that the number of private alleles was greater with ISSR markers.

The species-specific primer pair sets; CTF 5'-TCCCCTAAAAAGGACGTCTCCCG-3', CTR 5'-TCCCTCCGAATCCCAATGCGAGA-3'; CDF 5'-CCTCGGGACGCCCTCC-3', CDR 5'-CCCAATGCGAGGACGTTA-3' and CGF 5'-CAGCTCCGTCGCCCGGA-3', CGR 5'-TGTTTTACGGCAGGAGTC-3' targeting on ITS-rDNA were designed to use in PCR detection assay for *C. truncatum*, *C. dematium* and *C. gloeosporioides*, respectively. Species specificity of the primers was confirmed by successful detection of *Colletotrichum* spp. from infected legume plants, and the absence of DNA amplified products from other *Colletotrichum* species and divergent genus of the fungi. The results of sexual compatibility tests and genetical analysis showed the heterothallic nature of *Glomerella truncata*. In all the compatible crosses, perithecia, asci and ascospore morphologies were similar to those described earlier. However, the sexual

reproduction in *C. truncatum* has only been observed under laboratory conditions, and has never been reported in the field. The results indicated the intrinsic potential of *C. truncatum* for sexual reproduction by producing perithecia under natural conditions.



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**MORFOLOGI, PENCIRIAN MOLEKUL DAN PATOGENICITY
COLLETOTRICHUM SPP. DIKAITKAN DENGAN TANAMAN KEKACANG
BIJIRIN DI PENINSULA MALAYSIA**

Oleh

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Pengerusi: Profesor Madya Jugah Kadir, PhD

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Genus *Colletotrichum* adalah antara kumpulan kulat patogen yang paling penting bagi tumbuhan di dunia. Keperluan untuk diagnosis dengan tepat spesies ini adalah penting untuk menjamin kawalan penyakit yang berkesan dan membantu langkah-langkah kuarantin. Maklumat tentang populasi dan taburan spesies *Colletotrichum* pada tanaman kekacang bijirin di Malaysia adalah terhad. Sehingga kini tidak banyak maklumat yang diterbitkan mengenai biologi dan kepelbagaian genetik terutamanya kepada amalan pengurusan penyakit yang disebabkan oleh jangkitan kulat spesies ini. Matlamat kajian ini adalah untuk menerangkan ciri, biologi dan kepelbagaian spesies *Colletotrichum* yang dikaitkan dengan anthraknos kekacang. Jelasnya *Colletotrichum truncatum*, *Colletotrichum dematium* dan *Colletotrichum gloeosporioides* adalah spesies berbeza dengan *Colletotrichum* yang bertanggungjawab menyebabkan

anthraknos pada bijirin kacang yang diperhatikan di Malaysia dan didapati *Colletotrichum capsici* dan *C. truncatum* dikenali sebagai spesies yang unik, di mana ia menunjukkan *C. capsici* sebagai sinonim kepada *C. truncatum*. Sebahagian sifat morfologi yang diperoleh daripada piring kultur untuk kedua-dua spesies ini adalah saling bertindih. Tanpa mengira perumahannya, pemerincian *Colletotrichum* spp, bagi setiap pencilar mempamerkan darjah kepelbagaian yang besar dan saling bertindih dalam ciri-ciri morfologi dan ciri pada piring kultur. Taburan spesies ini berbeza mengikut asal-usul mereka. Data molekul yang diperolehi dari penjujukan lokus multigen DNA, analisis filogenetik, RAPD dan penunjuk identiti ISSR mengesahkan perbezaan spesies ini. Data molekul menunjukkan spesies *Colletotrichum* yang dikutip dari pelbagai jenis tumbuhan perumah adalah dari genetik yang berbeza. Tiada kesan geografi yang jelas telah diperhatikan. *C. truncatum* telah diketahui mampu menjadikan pelbagai spesies tanaman sebagai perumah seperti tanaman Fabaceae dan kubis (*Brassica chinensis*), cili (*Capsicum annuum*), tomato (*Solanum lycopersicum*), terung (*Solanum melongena*) dan salad (*Lactuca sativa*), dan sifat patogenik kepada spesies tumbuhan ini adalah ciri-ciri *C. capsici* yang sinonim kepada *C. truncatum*. Sebaliknya perumah bagi *C. dematium* dan *C. gloeosporioides* masing-masing terhadap kepada tanaman kacang buncis dan kacang soya, dan *Centrosema pubescens* sebagai perumah. *C. truncatum* telah dilaporkan lebih kerap menyerang tanaman kacang soya daripada tanaman lain di Malaysia. Berdasarkan hasil kajian ini, pencilar genetik yang patogenik ini sukar dibezakan daripada pencilar bukan patogenik. Data patogenik membuktikan struktur *C. truncatum* adalah berbeza dalam perumah yang berbeza, serta

data molekul menunjukkan persamaan dalam kepelbagaian genetik dan populasi perumah. Hasil kajian ini telah menunjukkan hubung kait yang jelas antara kepelbagaian genetik dan hubungan pencilar *Colletotrichum* spp. Data juga menunjukkan kepelbagaian genetik yang tinggi tersebar pada skala terkecil, sama ada di peringkat setempat dan perladangan.

Gabungan enam set data gen yang terdiri daripada 2771 aksara selepas penjajaran, 985 aksara parsimony informative. Parsimony dan neighbor-joining analisis menghasilkan topologi yang sama dan mengelompokkan semua pencilar kepada 3 kled ditakrifkan dengan sokongan bootstrap yang mewakili tiga spesies yang berbeza. Sebagai perbandingan, spesies epitip dari setiap takson telah dimasukkan dalam kajian filogenetik. Penjajaran jujukan mendedahkan kewujudan beberapa 'single nucleotide polymorphisms'(SNP) yang dikesan di lokus individu atau multigene *Colletotrichum* spp. pencilar daripada perumah tanaman yang berbeza dan kawasan geografi yang berbeza. ISSR dan penanda RAPD yang digunakan untuk mengkaji komposisi genetik spesies *Colletotrichum* daripada kekacang boleh digunakan untuk membezakan spesies ini. 'Molecular polymorphism' dalam dan di kalangan spesies *Colletotrichum* dinilai oleh RAPD dan penanda ISSR mendedahkan tahap perubahan molekul adalah lebih ketara perubahan yang dicirikan menggunakan 'multigene sequence analysis'. Walau bagaimanapun keputusan antara penanda adalah selari, tetapi disebabkan ini nisbah penunjuk berkesar adalah lebih besar dalam penunjuk ISSR. Pasangan primer spesies-spesifik menetapkan; CTF5'-TCCCCTAAAAGGACGTCTCCCG-3', CTR 5'-

TCCCTCCGAATCCCAATGCGAGA-3'; CDF 5'-CCTCGGGACGCCCTCC-3', CDR 5'-CCCAATGCGAGGACGTTA-3' dan CGF 5'-CAGCTCCGTCGCCCCGGA-3', CGR 5'-TGTTTTACGGCAGGAGTC-3' mensasarkan di ITS-rDNA direka bentuk untuk menggunakan di di pengesanan PCR dicerakinkan untuk *C. truncatum*, *C. dematium* dan *C. gloeosporioides*, masing-masing. Spesifisiti spesies daripada primers telah disahkan oleh ketiadaan produk DNA dari semua spesies selain daripada genus kulat *Colletotrichum* spp. Kaedah ini dapat membantu pengurusan penyakit tanaman secara mampan. Keputusan keserasian seks dan analisis genetik membuktikan sifat heterotalik daripada *Glomerella truncata*. Dalam semua keserasian silang, morfologi perithecia, askus dan askospora adalah sepadan seperti yang telah diterangkan sebelum ini. Walau bagaimanapun, pembiakan seks *C. truncatum* hanya diperhatikan di bawah keadaan makmal, dan tidak pernah dilaporkan dalam ladang. Keputusan menunjukkan potensi intrinsik *C. truncatum* untuk pembiakan seks menghasilkan perithecia di bawah keadaan semula jadi.

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I certify that a Thesis Examination Committee has met on **14 June 2013** to conduct the final examination of **Farshid Mahmodi** on his thesis entitled “**Morphological, Molecular Characterization and Pathogenicity of *Colletotrichum* spp. Isolated from Legume Crops in Peninsula Malaysia**” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the **degree of Doctor of Philosophy**.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

FARSHID MAHMODI

Date: 14 June 2013

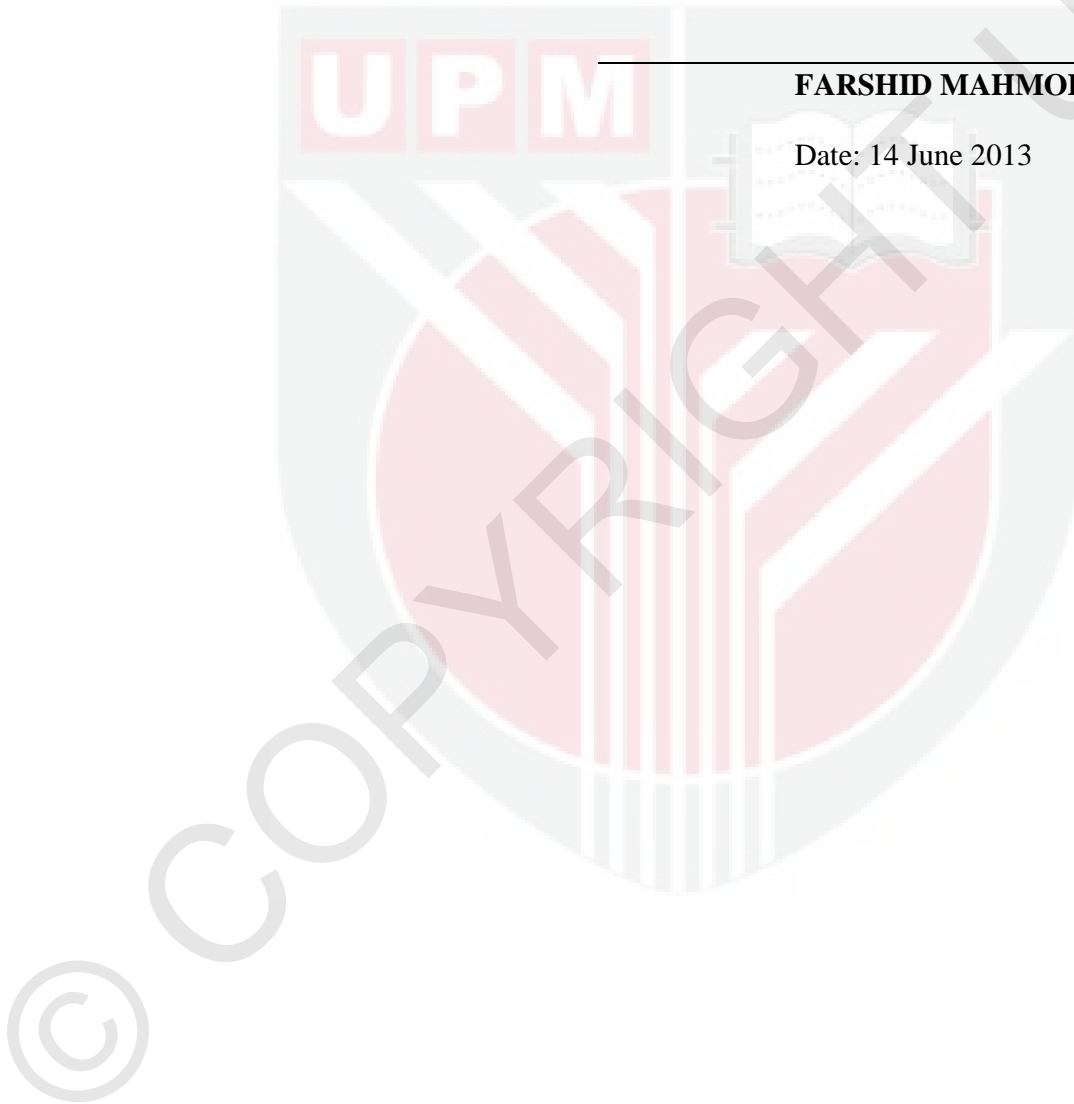


TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRAC	iii
ABSTRAK	vii
ACKNOWLEDGEMENT	xi
APPROVAL	xii
DECLARATION	xiv
LIST OF TABLES	xix
LIST OF FIGURES	xxii
LIST OF ABBREVIATIONS	xxvi
CHAPTER	
1 INTRODUCTION	1
2 LITERATUREREVIEW	5
2.1 Anthracnose Disease	5
2.2 <i>Colletotrichum</i> sp. Causal Agent of Anthracnose	6
2.3 Identification of <i>Colletotrichum</i> Species	8
2.4 Taxonomic History of <i>Colletotrichum</i>	9
2.5 Characteristics of <i>Colletotrichum</i> sp. Infections	13
2.6 Pathogenicity and Host Range of <i>Colletotrichum</i> Species	15
2.7 Teleomorph of <i>Colletotrichum</i> spp.	20
2.8 Mating System	22
2.9 Genetic Diversity	24
2.10 Molecular Techniques Based on PCR Amplification	26
2.10.1 DNA Sequencing	27
2.10.2 Random Amplified Polymorphic DNA (RAPD)	31
2.10.3 Inter Simple Sequence Repeat Marker (ISSR)	33

3	MORPHOLOGICAL CHARACTERIZATION, PATHOGENICITY AND HOST RANGE OF <i>Colletotrichum</i> spp. FROM LEGUME CROPS	36
3.1	Introduction	36
3.2	Materials and Methods	38
3.2.1	Isolation of <i>Colletotrichum</i> spp.	38
3.2.2	Colony Morphology	39
3.2.3	Colony Diameter and Growth Rate	40
3.2.4	Appressoria Formation	40
3.2.5	Pathogenicity Testing	41
3.2.6	Host Range	42
3.2.6.1.1	Greenhouse Experiments	43
3.2.6.1.2	Field Experiments	46
3.2.7	Statistical Analysis	47
3.3	Results	48
3.3.1	Morphological Characterization	48
3.3.2	Pathogenicity Testing	56
3.3.3	Host Range	60
3.3.3.1.1	Greenhouse Experiment	60
3.3.3.1.2	Field Experiment	63
3.4	Discussion	67
3.4.1	Morphological Characteristics	67
3.4.2	Pathogenicity and Host Range	71
3.5	Conclusions	76
4	MOLECULAR AND PHYLOGENETIC ANALYSIS BASED ON MULTI-GENE LOCI	77
4.1	Introduction	77
4.2	Materials and Methods	79
4.2.1	Isolation of <i>Colletotrichum</i> spp.	79
4.2.2	DNA Extraction	79
4.2.3	PCR Amplification	80
4.2.4	Gel Electrophoresis and Staining	82
4.2.5	Purification and Sequence Analysis	82

4.2.6	Phylogenetic Analysis	82
4.3	Results	85
4.3.1	Sequence Analysis of <i>Colletotrichum</i> spp. Isolates Based on ITS-rDNA Sequences	86
4.3.2	Phylogenetic Analysis	89
4.3.3	Sequence Analysis of <i>Colletotrichum</i> spp. Based on the β -Tubulin Gene	91
4.3.4	Phylogenetic Analysis	93
4.3.5	Sequence Analysis of <i>Colletotrichum</i> spp. Based on the Actin Gene	95
4.3.6	Phylogenetic Analysis	97
4.3.7	Sequence Analysis of <i>Colletotrichum</i> spp. Based on the Histone Gene	99
4.3.8	Phylogenetic Analysis	100
4.3.9	Sequence Analysis of <i>Colletotrichum</i> spp. Based on the Chitin Gene	103
4.3.10	Phylogenetic Analysis	106
4.3.11	Sequence Analysis of <i>Colletotrichum</i> Isolates Based on the GAPDH Sequences	108
4.3.12	Phylogenetic Analysis	110
4.3.13	Sequence Analysis of <i>Colletotrichum</i> spp. Based on the Multi-gene Sequences	112
4.3.14	Phylogenetic Analysis	112
4.4	Discussion	117
4.5	Conclusions	124
5	GENETIC DIVERSITY OF <i>COLLETOTRICHUM</i> SPP. ISOLATES USING RAPD AND ISSR MARKERS	125
5.1	Introduction	125
5.2	Materials and Methods	127
5.2.1	Fungal Isolates	127
5.2.2	DNA Extraction	128
5.2.3	Amplification and ISSR-PCR	128
5.2.4	ISSR Analysis	129

5.2.5	Amplification of RAPD-PCR	130
5.2.6	RAPD Analysis	131
5.2.7	Gel Electrophoresis and Staining	132
5.2.8	Index of Efficiency Rate of ISSR and RAPD Markers	132
5.3	Results	133
5.3.1	The ISSR-PCR	133
5.3.2	The RAPD-PCR	137
5.3.3	Index of Efficiency Rate of ISSR and RAPD Markers	141
5.4	Discussion	142
5.5	Conclusions	149
6	DESIGN OF SPECIES-SPECIFIC PRIMERS FOR DETECTING <i>COLLETOTRICHUM</i> SPP. ISOLATES	150
6.1	Introduction	150
6.2	Material and Methods	151
6.2.1	Fungal Isolates	151
6.2.2	DNA Extraction	152
6.2.3	PCR Amplification	152
6.2.4	Gel Electrophoresis and Staining	153
6.2.5	Species-Specific Primer Design	153
6.3	Results	156
6.4	Discussion	159
6.5	Conclusions	162
7	SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH	163
7.1	Summary and Conclusions	163
7.2	Recommendations for Future Research	169
	REFERENCES	170
	APPENDICES	193
	BIODATA OF STUDENT	255
	LIST OF PUBLICATIONS	256

LIST OF TABLES

Table	Page
2.1 Arx Von classification of <i>Colletotrichum</i> species (1957), including anamorph, teleomorph (when described), numbers of synonyms placed in each taxon, and host plant(s)	10
2.2 Major revisions of <i>Colletotrichum</i> presented by Arx Von (1957), Sutton (1980), Sutton (1992), Hyde <i>et al.</i> (2009b) and others	11
2.3 Infection strategies of <i>Colletotrichum</i> species on plant hosts	15
3.1 The list of plant species of Fabaceae family and other selected crops used for the host range studies under greenhouse and field conditions	43
3.2 Taxon, source and origin of <i>Colletotrichum</i> isolates used in this study	49
3.3 Representative morphological cultures of <i>Colletotrichum</i> spp. 10 days after incubation on PDA at 25 °C	50
3.4 Summary of morphological data for <i>Colletotrichum</i> species in the morpho-types	48
3.5 Cultural and morphological characters of <i>Colletotrichum</i> spp. on PDA, 10 days after incubation at 25 °C	55
3.6 Effect of <i>Colletotrichum</i> spp. on disease severity and incidence in the pathogenicity assay ^a to confirm virulence on the Fabaceae family	58
3.7 Effect of <i>Colletotrichum</i> spp. on disease severity and incidence in the host range assay ^a in greenhouse condition	62
3.8 Effect of <i>Colletotrichum</i> species on disease severity and incidence in the host range assay ^a under field condition	66
4.1 Primers used for PCR amplification and DNA sequencing	77

4.2 Isolates of <i>Colletotrichum</i> spp. associated with legume, with GenBank nos. accession of six genes	84
4.3. Distance matrix of ITS-rDNA sequences within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	88
4.4 Distance matrix of β -Tubulin sequences within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	92
4.5 Distance matrix of Actin gene sequences within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	96
4.6 Histone gene sequences derived from GenBank as a reference for phylogenetic analysis	100
4.7 Distance matrix of Histone gene sequences within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	101
4.8 Matrix distance of Chitin gene within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	105
4.9 Matrix distance of GAPDH gene within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	109
4.10 Matrix distance of multigene sequences within and among <i>C. truncatum</i> CT1-CT40, <i>C. dematium</i> CD42-CD44 and <i>C. gloeosporioides</i> CG45-CG50 isolates associated with legume crops	113
4.11 Descriptive tree statistics of Actin, Histone, GDPH, CHS I, β -tub2 and ITS	115
5.1 List of sequences of ISSR primers, their annealing temperatures and DNA polymorphisms which were employed to differentiate <i>Colletotrichum</i> species	129

5.2	List of sequences of RAPD primers, their annealing temperatures and DNA polymorphisms which were employed to differentiate <i>Colletotrichum</i> spp.	131
5.3	Matrix similarities derived from ISSR analysis within and among <i>Colletotrichum</i> spp. isolates associated with legume crops	136
5.4	Matrix similarities derived from RAPD analysis within and among <i>Colletotrichum</i> spp. isolates associated with legume crops	140
6.1	Sequence, temperature melting and rate of G/C % of species-specific designed primers	148



TABLE OF FIGURES

Figure	Page
2.1 Eukaryotic ribosomal DNA (rDNA) gene, showing primer locations in the ribosomal cassette consisting of the small subunit (SSU), large subunit (LSU), and internal transcribed spacers (ITS). [Gray boxes represent highly conserved regions. Primers are positioned above (forward primers) or below (revers primers) their sequence positions (White et al., 1991)]	28
2.2 β -Tubulin (tub2) gene showing primer locations in the tub2 gene cassette consisting of the coding and non-coding regions. Black boxes represent exon and wight boxes represent intron regions. Primers are positioned above (forward primers) or below (revers primers) their sequence positions	29
3.1 Following inoculation, the plants were covered with plastic bags for 48h and kept at the greenhouse condtions	45
3.2 The color of conidia mass in <i>C. gloeosporioides</i> (A); <i>C. truncatum</i> (B); <i>C. capsici</i> (C), and <i>C. dematium</i> (D) on PDA culture	53
3.3 Host range assay using <i>C. truncatum</i> isolate CTM4 under field conditions (Ladang Dua, UPM, Selangor). [A) early growth stage of plants, B) disease symptoms on lima bean, C) plotting of experimental design, D) anthracnose on beans, E) disease symptoms on lentil, F) anthracnose on soybean	57
3.4 Pathogenicity assay using <i>Colletotrichum</i> spp. isolates in vitro conditions. Disease symptoms on A) soybean (CTM4), B) eggplant (CTM4), C) bean (CTM8), D) chilli (CTM4), E) faba bean (CTM4), F) lima bean (CTM12)	60
3.5 Host range assay using <i>C. truncatum</i> isolate CTM4 in greenhouse conditions. A) plants before inoculation, B) covering plants with the plastic bags for 24 after inoculation, C) placing plants on benches after 24 h, D) disease symptoms on chickpea, E) disease symptoms on cowpea, F) anthracnose on soybean	64
4.1 Gel electrophoresis of PCR products from representative isolates of <i>Colletotrichum</i> spp. to compare the size of amplified multigene loci of β -tubulin (~780 bp), actin (~290 bp), histone (~420 bp), GPDAH (~300 bp), ITS-rDNA (~620 bp) and chitin (~300 bp), respectively	85

- 4.2 Gel electrophoresis showing representative bands of PCR products from ITS-rDNA region (~620 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50 are *C. gloeosporioides* isolates 86
- 4.3 Maximum parsimony phylograms inferred from ITS-rDNA sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legume crops and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 90%. The tree is rooted with *Colletotrichum lindemuthianum* (CBS 151.28) 90
- 4.4 Gel electrophoresis showing representative bands of PCR products from β -Tubulin gene (~800 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50 are *C. gloeosporioides* isolates 91
- 4.5 Maximum parsimony phylograms inferred from β -Tubulin sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 90%. The tree is rooted with *Colletotrichum lindemuthianum* (CBS 151.28) 94
- 4.6 Gel electrophoresis showing representative bands of PCR products from Actin gene (~310 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50 are *C. gloeosporioides* isolates 95
- 4.7 Maximum parsimony phylograms inferred from Actin gene sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 99%. The tree is rooted with *Colletotrichum lindemuthianum* (CBS 151.28) 98
- 4.8 Gel electrophoresis showing representative bands of PCR products from Histone gene (~440 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50; are *C. gloeosporioides* isilates 98

- 4.9 Maximum parsimony phylograms inferred from Histone gene sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 99%. The tree is rooted with *Colletotrichum lindemuthianum* (CBS 151.28) 102
- 4.10 Gel electrophoresis showing representative bands of PCR products from chitin gene (~320 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50 are *C. gloeosporioides* isolates 103
- 4.11 Maximum parsimony phylograms inferred from Chitin gene sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 99%. The tree is rooted with *Colletotrichum lindemuthianum* (CBS 151.28) 107
- 4.12 Gel electrophoresis showing representative bands of PCR products from GAPDH gene (~300 bp) of *Colletotrichum* spp. isolates. Lane M 100 bp marker, Lanes CT1-CT40 are *C. truncatum* isolates, lanes CD42-CD44 are *C. dematium*, lanes CG45-CG50 are *C. gloeosporioides* isolates 108
- 4.13 Maximum parsimony phylograms inferred from GAPDH sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 99%. The tree is rooted with *Colletotrichum lindemuthianum* 108
- 4.14 Maximum parsimony phylogram inferred from multigene sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from legumes and selected sequences of *Colletotrichum* species (epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap above 90%. The tree is rooted with *Colletotrichum lindemuthianum* 114
- 5.1 Gel electrophoresis of amplification products from *Colletotrichum* spp. 131

- genomic DNA obtained by ISSR primers UBC 808, UBC 834, UBC 810 and UBC 112, respectively. The 1st and the last lanes (M) are 2-log DNA ladder. Lanes CT1-CT40 are the representative isolates of *C. truncatum*, lanes CD42-CD44 are the representative isolates of *C. dematium*, lanes CG45-CG50 are the representative isolates of *C. gloeosporioides*
- 5.2 Dendrogram showing the genetic relationships among representative isolates of *Colletotrichum* spp. [derived from Unweighted Pair-Group (UPGMA) cluster analysis, based on Jaccard's similarity coefficient] 135
- 5.3 Gel electrophoresis of amplification products from *Colletotrichum* spp. genomic DNA obtained by RAPD primers OPA-11, OPA-01, OPJ-20 and OPA-09, respectively. The 1st and the last lanes (M) are 2-log DNA ladder. Lanes CT1-CT40 are the representative isolates of *C. truncatum*, lanes CD42-CD44 are the representative isolates of *C. dematium*, lanes CG45-CG50 are the representative isolates of *C. gloeosporioides* 138
- 5.4 Dendrogram showing the genetic relationships among representative isolates of *Colletotrichum* spp. [derived from Unweighted Pair-Group (UPGMA) cluster analysis, based on Jaccard's similarity coefficient] 139
- 6.1 DNA sequence comparison of the internal transcribed spacer (ITS) region between *C. truncatum* (CM1), *C. dematium* (CD41) and *C. gloeosporioides* (CG49). The location of the species-specific primer sequences are delimited in boxes 157
- 6.2 Amplicon generated with species-specific primer CTsF/CTsR and amplified under optimal PCR conditions (Annealing temperature 54 °C). [(A); Lane M 100 bp marker, Lane 1 *C. truncatum*, lane 2 *C. dematium*, lane 3 *C. gloeosporioides*, lane 4 *Diaporthe phaseolorum*, lane 5 *Fusarium solani*, lane 6 *Fusarium oxysporum* fsp. *ceceri*, lane 7 *Fusarium equiseti*, lane 8 *Alternaria tenuissima*, lane 9 *Stemphylium solani* correspond to genomic DNA isolated from other *Colletotrichum* species and heterogeneous genus of fungi and lane 10 PCR negative control (distilled water). (B); Sensitivity of a designed primer pairs CTsF/CTsR for detection of *C. truncatum*. 10-fold serial dilution of genomic DNA extracted from in vitro culture; [Lane M 100 bp marker, Lane 1 undiluted 100 ng/μl, Lane 2 10 ng/μl, Lane 3 1 ng/μl, Lane 4 100 pg/μl, Lane 5 10 pg/μl, Lane 6 1 pg/μl, Lane 7 100 fg/μl, Lane 8 PCR negative control (distilled water). Markers indicate the ~400 bp amplified product that is unique to *C. truncatum* isolate CTM16] 158

LIST OF ABBREVIATIONS

°C	Degree Celsius
spp.	Species (plural)
CRD	Complete Randomized Design
LSD	Least Significant Difference
PDA	Potato Dextrose Agar
CTAB	Hexadecyltrimethyl-Ammonium Bromide
ddH ₂ O	Double Distilled Water
M	Molar
DNA	Deoxy Ribo Nucleic Acid
Uv	Ultra Violet
EB	Ethidium Bromide
v	Volt
EDTA	Ethylene Diamine Tetraacetic Acid
TAE	Tris Acetic Acid EDTA
TE	Tris EDTA
µl	Microliter
mM	Milimolar
µM	Micromol
ng	Nanogram
pg	Picogram
fg	Festogram
rpm	Revolutions Per Minute
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
bp	Base Pair(s)
NCBI	National Center Biotechnology information
ISSR	Inter Simple Sequence Repeat
RAPD	Random Amplification of Polymorphic DNA
EMR	Eeffective Marker Ratio
ITS	Interal Transcribed Spacer
GAPDH	Glycer Aldehyde 3, Phosphate DehHdrogenase
B.C	British Columbia
BLASTn	Basic Local Alignment Search Tool Nucleotide

v/v
w/v
U.K
USA

Volume/Volume
Weight/Volume
United Kingdom
United States of America



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CHAPTER 1

INTRODUCTION

Legumes belong to the Fabaceae family, which comprise of 750 genera and 16,400 species of herbs, shrubs, trees and climbers. Traditionally, the legume family has been divided into three subfamilies; *Caesalpinieae*, *Mimosoideae* and *Papilionodeae* (Luckow *et al.*, 2003; Singh *et al.*, 2007a). Legumes are the second most important crop plants after cereals in their importance based on area harvested and total production. They have been grown in about 190 million hectares, and the world's production is approximately 300 million tons worldwide (Graham and Vance, 2003). Seeds of grain legumes contain at least 20 to 40% of protein. Grain legumes are rich in protein, complex carbohydrates and essential mineral nutrients (Wang *et al.*, 2003). In many parts of the world, legumes are a dependable cash crop for smallholder farmers due to their adaptation to marginal soil conditions, and their ability to biologically fix nitrogen (Hillocks *et al.*, 2006). Legumes have a unique ability of benefitting cropping systems by forming symbiotic associations with *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* bacteria which are able to reduce atmospheric N into ammonia (Hopkins and Hunter, 2004).

Grain legumes are also rich sources of essential vitamins, minerals and important amino acids. The use of legume crops has helped in preventing cardiovascular diseases, strokes, Parkinson's disease, Alzheimer's disease and even cancer (Singh *et al.*,

2007a). Main dietary legumes include soybean (*Glycin max*), which is used in multiple applications in the food and seed industries; peanut (*Arachis hypogea*); cowpea (*Vigna unguiculata*); common bean (*Phaseolus vulgaris*); chickpea (*Cicer arietinum*); pigeon pea (*Cajanus cajan*); lentil (*Lens culinaris*); mung bean (*Vigna radiata*); and faba bean (*Vicia faba*); all of which contribute significantly to the diets of large numbers of people in Asia, Africa and South America (Lodwig and Pool, 2003). Using of legume in Malaysia as ground covers (Kacangan) have been considered in rubber and oil palm plantation for soil stability, weed suppression and fertility. In Malaysia three species of legume plants, *Pueraria phaseoloides*, *Calopogonium caeruleum* and *Centrosema pubescens* are widely cultivated as ground covers in rubber and oil palm plantations. These traditional leguminous covers successfully compete with volunteer weed growth, particularly *Mikania*, *Asystasia* and grasses without the need for laborious hand weeding or expensive weeding with herbicides during establishment (Wahab, 1997). The production of the majority of legume crops grown all over the world are heavily constrained by a number of biotic (drought, freezing, salinity) and biotic (fungi, bacteria, nematodes, viruses, insects, weed plants) stresses, especially in the tropical and subtropical areas of the world (Lodwig and Pool, 2003; Dwivedi *et al.*, 2005). Anthracnose caused by *Colletotrichum* species is one of the most common diseases of legumes that attack the grain and foliage causing serious yield losses in several parts of world. The disease is most common and severe on legumes such as soybean, common bean, lentil, cowpea, lima bean, groundnut and faba bean. It frequently causes significant economic losses in annual crops such as legumes, cereals, and vegetables at all stage of their development, i.e. from seedling to mature plants (Sartorato, 2002).

Colletotrichum species and its sexual state *Glomerella* cause anthracnose symptoms on many annual plants and also are the causal agents of canker, dieback, and rot on some perennial plants (Freeman *et al.*, 2000; Agrios, 2005). The important aspects of *Colletotrichum* spp. include worldwide distribution regardless of differences in climate, from temperate to tropical and subtropical regions, infections and disease occurrence at any stage of plant growth, thus anthracnose can occur either in the field or in the greenhouse and storage room, pathogen has broad host range on plants including annual and perennial plants and pathogen can infect the entire plant, including underground (roots) and aboveground parts (Freeman *et al.*, 2000). Different disease symptoms may appear at once in different parts of plants e.g. leaf, stem, pods and roots, following the infection of *Colletotrichum* species (Lenne, 1992).

There have only been a few dispersed studies on the causal agents of anthracnose disease on legumes in Malaysia. The literature search indicated that no thorough investigations have ever been carried out on the causal agent of anthracnose on legumes in Malaysia. Therefore, studies on *Colletotrichum* species associated with anthracnose of legume crops in Malaysia are addressed in this thesis. In addition, the genetic diversity of *Colletotrichum* spp. populations from diverse host plants of legumes and different geographical region was investigated. The results of the present study should provide better insights into the biology and etiology of the pathogen and this will be helpful in the development of better disease management strategies and in the breeding of resistant varieties for legume production in Malaysia. The research focused on determining the biology and diversity of *Colletotrichum* associated grain

legumes in Malaysia. Therefore, this research was undertaken with the following specific objectives:

- i) To assess phenotype variability among monoconidial isolates using cultural and morphological characteristics
- ii) To evaluate the pathogenicity and host range among *Colletotrichum* spp. isolates in *vivo* and in *vitro* conditions;
- iii) To determine genetic relatedness among *Colletotrichum* spp. isolates by phylogenetic and sequence analysis using multigene loci; including actin, chitin, β -tubulin (tub 2), histone, glyceraldehyde-3-phosphate dehydrogenase (GPDH) and the rDNA internal transcribed spacer (ITS);
- iv) To examine genotype diversity among *Colletotrichum* spp. isolates using Randomly Amplified Polymorphic DNA (RAPD) and Internal Simple Sequence Repeats (ISSR);
- v) To design species-specific primers in order to rapidly detect of *C. truncatum*, *C. dematium* and *C. gloeosporioides* isolates from diverse host plants.

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