MORPHOLOGICAL CHARACTERIZATION, MOLECULAR IDENTIFICATION AND PATHOTYPING OF *COLLETOTRICHUM* SPECIES IN PENINSULAR MALAYSIA

KHOR BEE SYM

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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UNIVERSITY OF MALAYA
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2013

ABSTRACT

Nineteen isolates of *Colletotrichum* spp were obtained from infected fruits, 16 isolates were from *Capsicum annuum*, and three isolates from non-host of *Capsicum annuum*, which consist of one isolates from papayas and two isolates from strawberries. All showing anthracnose symptom. Growth rate can be the criteria for *Colletotrichum* identification. *C. gloeosporioides* has the fastest growth rate among the 3 species and *C. acutatum* has the slowest growth. *C. truncatum* has the average growth rates. ANOVA results showed that all of growth rate showed that there are significant different among the species. *C. truncatum* has the longest length of conidia (falcate) size and *C. acutatum* (fusiform) has the shortest length of conidia size. *C. gloeosporioides* (cylindrical) has the widest width compared to *C. truncatum*.

ITS has identified six isolates as *C. truncatum*, five isolates of *C. scovillei* and five *C. gloeosporioides* complexes most likely as one *Colletotrichum species, one C. asianum, one C. siamense* and two C. *queenslandicum*. More gene sequencing work needed to clearly differentiate the species besides ITS region.

Pathotypes studies have identified on wounded mature fruits were one pathotype on *C. scovillei* which were highly virulent on the genotype *Capsicum annuum* (Kulai) with host reaction of 7, three pathotypes were identified within *C. truncatum* infecting (Kulai and bell pepper) with host reaction of 5 to 9, four pathotypes were identified within *C. gloeosporioides* complex with only two isolates infecting Kulai and bell pepper, one isolates of *Colletotrichum* sp. and two isolates infecting only Kulai. All isolates from *C. annuum* used in this study showed no infection on the resistant genotypes *C. chinense*. Non-host *Colletotrichum* species were able to infect only the genotype Kulai.

ABSTRAK

19 isolat *Colletotrichum* spp telah diperolehi daripada buah cili yang dijangkiti penyakit antraknos buah. 16 isolat dari *Capsicum annuum*, satu isolat dari betik dan dua isolat daripada strawberi. Semua anthracnose menunjukkan simptom. *C. gloeosporioides* mempunyai kadar pertumbuhan terpantas di antara tiga spesies dan *C. acutatum* mempunyai pertumbuhan perlahan. *C. truncatum* mempunyai kadar pertumbuhan purata. Analysis ANOVA menunjukkan bahawa semua kadar pertumbuhan menunjukkan bahawa terdapat perbezaan yang signifikan di kalangan 3 spesies. *C. truncatum* mempunyai 'length' terpanjang dan saiz konidia (berbentuk sabit) dan *C. acutatum* (fusiform) mempunyai 'length' terpendek saiz konidia. *C. gloeosporioides* (silinder) mempunyai 'width' terluas berbanding *C. truncatum*. Terdapat korelasi antara kadar pertumbuhan koloni dan saiz conidial.

ITS telah mengidentifikasikan enam isolate *C. truncatum*, lima isolat *C. scovillei* dan lima *C. gloeosporioides* kompleks yang terdiri daripada sate *Colletotrichum* species, satu *C. asianum*, satu *C. siamense* dan dua *C. queenslandicum*. Lebih gen perlu dikaji untuk identifikasi yang tepat selain daripada ITS.

Kajian pathotype pada buah yang matang mendapati bahawa satu pathotype adalah dari *C. scovillei* yang menunjukkan jangkitan hos 7 pada kulai, tiga pathotype didapati daripada *C. truncatum* yang menjangkiti hanya (Kulai dan bell pepper) dengan jangkitan hos dari 5 hingga 9. Empat pathotype didapati daripada kompleks *C. gloeosporioides* dengan satu hanya satu isolate *C. asianum* yang mengjangkiti kulai dan bell pepper, dan satu isolat *Colletotrichum* sp. dan dua isolat *C. queenslandicum* hanya dapat mengjangkit pada Kulai, Semua isolat yang dikaji tidak menunjukkan jangkitan pada *C. chinense. Colletotrichum* isolat daripada hos lain hanya dapat mengjangkiti Kulai.

ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor, Associate Professor Dr. Md. Yusoff Musa, Dr Mohammed Rizman Idid and Professor Dr. Paul Taylor for offering me this project and their invaluable advises and guidance throughout the course of this research.

I would like to thank the administrative staff of IPS, University Malaya for the facilities provided and help. This project was funded by the Postgraduate Research Grant (PPP) grant number PS157/2008A and PS370/2009B. A vote of thanks to the state Department of Agriculture for their kind helped in arranging the sampling location for this study. This work would never be possible with the help of farmers that allow us to do the sampling. I'm really indebted to their kindness and co-operation during our field trips, En. Rosli Halip and En Zul.

I would like to thank my dearest colleagues, Saw Teng and Ami for their help in this study. Last but not least, the deepest appreciation goes to my parents who support me endlessly. Special heartfelt also goes to my husband for his moral support and guidance.

Thank you very much!

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µm	micrometer
BLAST	Basic Local Alignment search Tool
bp	Base pair
ITS	Internal Transcribed Spacer
LSU	Large subunit
MCMCMC	Metropolis-coupled Markov Chain Monte Cralo
MEGA	Mega Basic Local Alignment search Tool
PAUP	Phylogenetic Analysis using Parsimony
PCR	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
SSU	Small subunit
TBE	Tris-Borate-EDTA
TBR	Tree Bisection Reconnection
TrN	Tamura and Nei

Chapter 1 General introduction

1.1 Anthracnose disease by *Colletotrichum*

Colletotrichum is one of the most important plant pathogens that caused disease on a wide variety of tropic and subtropic woody and herbaceous plant (Sutton, 1992; Hyde *et al*, 2009a). According to Dean *et al*, (2012), *Colletotrichum* was the eighth most important group of plant pathogenic fungi in the world. As plant pathogens, they primarily caused anthracnose disease on economically important crops around the world that reduces post-harvest production due to fruit infection (Phoulivong *et al*, 2010). *Colletotrichum* not only caused diseases on chilli plant (*Capsicum* spp) but also to guava (*Psidium guajava*), jujube (*Zizyphus mauritiana*), mango (*Mangifera indica*) and papaya (*Carica papaya*) (Damn *et al*, 2009; Freeman *et al*, 1996).

The disease is usually in quiescence which does not develop until the chilli fruit ripens. Anthracnose may occur on leaves, stems and both pre and post-harvest fruits. Typically, symptoms that appear firstly on mature fruits are small, water-soaked and sunken lesions that rapidly expand. Fully-expanded lesions are sunken and range from dark red to light tan.

Chilli is from the genus *Capsicum* was originated from the American tropics and has been propagated throughout the world (Pickersgill, 1997). *Capsicum* has variety of names such as chilli, chilli pepper or pepper depending on place and type of fruits. This genus has about 20-27 species, whereby the 5 most common 5 species that have been domesticated as *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*, and cultivated in different parts of the world. *C. annuum* is one of the most common cultivated crops worldwide (Tong & Bosland, 1999) and many breeding programs have concentrated on the non-pungent *C. annuum*. Chilli is widely used in culinary for its

nutritional value, aroma, texture and flavour. Chilli comprises of steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins A and C, protein, fibre and mineral elements, such as potassium and folic acid.

1.2 Systematics of *Colletotrichum*

Colletotrichum was first described by Tode in 1790 (Hyde *et al*., 2009a; Sutton, 1992) in the genus *Vermicularia* and later studied by von Arx in 1957 and reduced the number of described taxa from 750 species to 11 species (within a total of 23 accepted specific and infraspecific taxa) in the *Colletotrichum* taxonomy. There are about 600 synonyms of this species (von Arx, 1957). *Colletotrichum* leads its life as an endophyte, saprophyte, or phytopathogen (Hyde *et al*., 2009a). There have been much confusion and misunderstanding in the nomenclature of *Colletotrichum* species. *Colletotrichum* species have been described and named through morphological, molecular and pathogenicity studies. Their species identification is difficult because there are few distinct morphological characteristics, teleomorphs are rarely formed and morphological variations occur in cultures and host-pathogen relationships. These taxonomic uncertainties also prevent accurate diagnosis of the disease, developing proper control strategies and quarantine programmes (Hyde *et al*., 2009b). However, at present, the combination of molecular tools with morphological studies is a good approach to study the *Colletotrichum* species (Cai *et al*., 2009).

Species identifications of some species in this study were based on a basic reference of morphological characteristics by Sutton (1980b). Fungal classification relies mostly on morphological appearance and this is a phenotypic approach. For example, not all morphological features were considered as equally important. Some characters were viewed more important than others (Guarro *et al*., 1999). However, description of conidial shapes are subjective, not standardized and may be influenced by the

environment. Thus, morphological characteristics often received criticisms for such flaws. These structures were often disregarded as they required a combination of skill, intuitiveness and experience to describe them accurately (Guarro *et al*., 1999).

1.3 Taxonomic ambiguities of *Colletotrichum* spp identification

The genus *Colletotrichum* has created much confusion in its taxonomy and nomenclature (Hyde *et al*., 2009b). The identification within the genus is complicated because the teleomorphic stages are rarely observed. It is important to have a stable taxonomy of *Colletotrichum* species. Hyde *et al*. (2009a) stated that there are 66 names of *Colletotrichum* in common use, and 19 recently used names are put into a doubtful category, together with information on host diseases and their characteristics.

Although morphology is not an adequate identification criteria due to the variations which are strongly influenced by environmental factors (Than *et al*., 2008), such morphological characteristics such as conidial shape may provide some information to pre-identify the isolates. In our study, molecular genetic tecniques were incorporated to confirm the identity of the isolates because molecular characteristics such as DNA sequences are more consistent and not easily influenced by environmental pressure even when evolution occurs. A combination of multigene analysis and morphological characters creates an accurate identification of *Colletotrichum* (Hyde *et al*., 2009b). However, molecular identification also has its limitations, thus the genetic analysis requires reliable gene sequencing or GenBank data in order for identification to be certain and does not show great divergences among compared sequences. Therefore, sequences of epitypes are required to clarify and rectify the ambiguous data (Hyde *et al*., 2009b).

Colletotrichum capsici (Syd.) E.J. Butler & Bisby, (1931) with falcate-spore characteristic that been recently categorized in recently used names which are regarded as doubtful. However, *Colletotrichum capsici* is now known as *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore (1935), based on the studies on Australian isolates using Internal Transcribed Spacer and β-tubulin genes (Damm *et al*., 2009). It is important to have a standard to link between phylogenetics and pathogenicity for the identification of species. ITS sequences play a powerful role in phylogenetic tree construction but it is still questionable to whether they will provide an adequate resolution for the determination and differentiation of *Colletotrichum* species. According to Cai *et al*. (2009), 343 ITS sequences of tentative named *Colletotrichum gloeosporioides* isolates were analyzed and found that the ITS sequence data showed more than 86% sequence divergence from *C. gloeosporioides* epitype. Inclusion of type specimen is crucial for species comparison and phylogenetic analyses. ITS sequences are useful for the construction of interspecific relationships but not for intraspecific relationships. In Cai *et al*. (2009), a standard protocol was used to study *Colletotrichum* species, which comprised of several approaches, including isolation, morphological studies, multilocus phylogeny, growth rate comparisons, biochemical and pathogenicity tests. Through such protocols, scientists are able to compare and distinguish *Colletotrichum* species

Epitypification is important to provide a material that represents the type of species or referral specimen for species identification. An epitype is crucial to replace the lost material or poor condition types. A good epitype helps in the understanding of a species and stabilizes the taxonomic problems (Hyde, 2008). Epitypification fixes a name to the specimen, which is crucial for the phylogenetic studies of a taxon. Thus,

misidentification causes difficulties to scientists in diagnosing and implementing the control measures to overcome the diseases caused.

1.4 Identification of *Colletotrichum* characteristics

Many studies have been conducted on *Colletotrichum* throughout the years. Traditionally, *Colletotrichum* species were identified using morphological characteristics based on the shapes of conidia, appresoria, presence or absence of setae, scloretia, acervuli and teleomorph stage, and cultural characters which included colony colour, growth rate and texture (Pamela *et al*., 1992; Sutton, 1980a). A summary of some characters need to differentiate among *Colletotrichum* species is shown in **Table 1.1**.

Table 1.1. Differences of morphological features for the taxonomic identification of *Colletrichum* spp.

Morphological Feature	*C. truncatum* (Schwein.) Andrus & W. D. Moore	*C. acutatum* (Simmonds ex Simmonds)	*C. gloeosporioides* (Penz.) Penz. and Sacc.
Culture colony character on Potato Dextrose Agar	White to grey. Most showed diurnal zonation of dense and sparse development of aerial mycelium, with the dark green center and the cottony mycelium.	Pale orange colonies with little aerial mycelium and a few orange conidial masses around the centre.	Pale grey to black zonated colonies with abundant orange conidial masses near the centre. Some showed diurnal zonation of pale grey to black aerial mycelium, whilst others produced aerial mycelium in an even, felted mat.
Growth rate	7.1 mm day^{-1}	5.8 mm day^{-1}	11.1- 11.2 mm day^{-1}
Conidia shape morphology	Falcate, fusiform, gradually tapered towards each end.	Straight, fusiform (average 80% occurrence), slightly tapered at each end, slight medianly constricted.	Straight, cylindrical, apex obtuse, base truncate.
Conidia Size (μm)	16-25 x 3-4	8.5-16.5 x 2.5-4	6–25 x 3.5-6
Conidia colour	Pale buff to salmon masses	Rose, salmon pink to orange masses	Greyish white to dark grey
Sclerotia	Absent	-	Absent

*Source: (Cano *et al*., 2004; Shenoy *et al*., 2007a; Sutton, 1992; Than *et al*.,2008)

1.5 Molecular genetic techniques and the phylogenetics of *Colletotrichum spp.*

Molecular genetic techniques have been applied to compensate the limitations of morphological identifications and to answer the unresolved and doubtful questions in *Colletotrichum* studies. The advent of PCR based techniques has enabled analysis of nucleotide sequences or genetic markers from small amount of resources.

One of these techniques is the DNA sequencing method that obtains DNA sequences of genes or regions used to identify phylogenetic relationships. The most frequently used group of genes is rRNA, protein introns of encoding genes, such as β-tubulin (O' Donnell, 1992), actin (Cox *et al*., 1995), chitin synthase (Bowen, *et al*., 1992), acetyle coenzyme A synthase (Birch *et al*., 1992), and glyceraldehydes-3-phosphate dehydrogenase (Harmsen, *et al*., 1992) for fungal studies.

In recent decades, molecular markers have been widely used to measure the variation in pathogen populations (Brown *et al*., 1996; Milgroom, 1996; McDonald, 1997). Genetic markers such as Random amplified polymorphic DNA (RAPD) markers, simple sequence repeat (microsatellite), inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) technique, and genomic DNA RFLP technique, are among the most frequently used. RAPD has the disadvantage of not distinguishing whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies) and requires heavy laboratory dependent and strict protocols for results to be reproducible. Mismatching between primer and template may result in absence of PCR product and RAPD results can be difficult to interpret.

AFLP is highly reproducible with better, resolution and sensitivity compared to other techniques, and has the capability to amplify between 50 and 100 fragments at one time. AFLP were widely used for the identification of genetic variation for closely

related species of plants, fungi, animals, and bacteria due to its capability to detect various polymorphism from different genomic regions simultaneously.

Simple sequence repeats (microsatellites) and inter simple sequence repeat, (ISSR) which is a genome region between microsatellite loci, are markers that maybe conserved or non-conserved region and may not be useful in distinguishing individuals but may delinmite species. Microsatellite sequencing and ISSR sequencing are mutually assisting and producing primers for the PCR amplification of each other.

Other than DNA sequencing, the RAPD technique have been widely used to study *Colletotrichum* species (Ratanacherdchai *et al*., 2010). Generally, sequencing of ITS region has been widely applied for species identification. The ITS region comprises of ITS 1, 5.8S and ITS2 spacers. It is a non-protein coding region and is a popular choice for identification and phylogenetic studies of fungi because of the existence of multiple copies in the fungal genome. It has high sequence variations thus is able to differentiate microfungal isolates to the species level. The ITS regions were frequently used in molecular identification of fungi, hence the database are very established that would give good interpretation.

1.6 *Colletotrichum* systematic on *C. gloeosporioides* and *C. acutatum* complexes

Numerous studies have reported on subgroups within the *C. acutatum* (Damm et al., 2012) and *C. gloeosporioides* (Weir *et al.*, 2012) species complex. In the complexes, there were highly diverse in phenotype and have specific host or geographical regions. Multilocus molecular analysis (ITS, ACT, TUB2, CHS-1, GAPDH, HIS3) were the basic of species recognition, eventhough morphological and cultural characters that allow alternative means of species recognition were given whenever where possible (Weir *et al*., 2012). In the studies of Damm *et al.* (2012) and Weir et al. (2012) stated

that previously identified *C. acutatum* and *C. gloeosporioides* have confirmed that molecular groups previously recognised and identified a series of novel taxa. There were 22 species plus one subspecies within the *C. gloeosporioides* complex and 31 species were accepted and 21 which have not been previously within the *C. acutatum* complex.

1.7 Phylogenetic concept

Phylogenetic analysis aims to trace the evolutionary history of organisms. Molecular phylogenetic analysis utilizes sequence data to produce phylogenetic trees to infer relationships and investigate character changes. Phylogenetic trees can also help in identification of species.

Phylogenetic trees commonly used are distance (Neighbour Joining) and discrete (Maximum parsimony, maximum likelihood) methods. Distance trees such as Neighbour-Joining (NJ) tree creates the trees based on distance data which is the overall differences for every pairwise comparison of sequences. The distance calculated after every pairwise comparison is clustered by the neighbour-joining method (Saitou & Nei, 1987) of which they form individual clades of closely related sequences. The confidences of branching patterns obtained were tested by using the bootstrapping analysis (Efron & Tibshirani, 1993; Kishino & Hasegawa, 1989).

Discrete methods such as Maximum Parsimony & Maximum Likelihood, consider the changes of each nucleotide site in sequences directly. The most parsimonious tree is the tree with the shortest length with the least amount of changes. This rearrangement would result in huge set of trees with different topologies using heuristic method to find the most parsimonious tree, often sampling only a subset of all possible topologies to obtain the closest tree to the shortest or parsimonious tree

(Maddison, 1991). Bootstrap analysis is then carried out to evaluate the confidence of the tree.

Another method is the Bayesian analysis which aims to find the most likely tree by sampling a subset of trees that have the highest posterior probability (Huelsenbeck *et al*., 2001), calculated by integrating the prior probability of parameters with the likelihood of our tree. The random sampling of our parameters will give a large set of trees with different posterior probability values. In order to sample trees with only the highest posterior probability, Markov chain Monte Carlo (MCMC) algorithm is used. The topologies of trees with the highest posterior probabilities are summarized into a consensus tree, giving our Bayesian tree with branch support expressed as posterior probability values. The limitations of Bayesian analysis stemed from its many assumptions providing far too many possible topologies and this process can be extremely time consuming. A solution to this problem would be to specify the parameters as selected from jModeltest.

We have discussed the three different methods (distance, parsimony and Bayesian) to estimate the evolutionary history or phylogeny pathway of microfungi. However this phylogenetic tree is merely an approximation of the actual evolutionary history, whereby the 'true' tree will never be known. As mentioned by Posada and Buckley (2004), 'All phylogenies are wrong but some are useful'

1.8 Pathotyping studies of *Colletotrichum*

According to Taylor and Ford (2007), a pathotype can be defined as a subclass or group of isolates distinguished from others of the same species by its virulence on a specific host (genotype) i.e., a qualitative difference in disease severity. In contrast, aggressiveness reflects the natural variation in virulence or level of disease (measured

quantitatively) within the pathogen population. Recent studies in Thailand identified three pathotypes for both *C. truncatum* and *C. acutatum* isolates which were able to infect resistant genotypes of *Capsicum chinense* – PBC932 and *Capsicum baccatum* - PBC 1422, respectively (Taylor, 2007). Taylor & Ford (2007) stated that confusion might happen as whether the true pathotypes differences do exist, or whether the differences of observation in disease severity are a measure of the natural distribution of aggressiveness within a population, ranging from low to high. However, the level of aggressiveness of isolates is important when considering development of resistance in breeding programmes and disease control management. Genotypes with partial resistance would result in lower level of infections that eventually will decrease the amount of inoculum in field, hence limiting the potential of epidemics.

Bioassay for pathotyping can provide important biological information when evaluating individual strains for the range of virulence within a genetically diversified species. Molecular data combined with pathotyping work could provide greater insight about the maintainence of intraspecific variation when compared with isolation techniques (Ehler *et al*., 2004).

In pathogenicity tests, infection might happen on a variety of host plants or cultivars that previously have not been infected by the pathogen, or the levels of virulence may be milder or more severe than previously observed. A resistant genotype may escape from the current prevalent pathogen but may be challenged by a new parasitic strain with a virulent gene and is capable of overcoming that resistance (Than *et* al, 2008). Pathotypes of *Colletotrichum* species were determined via their potential of infection on various *Capsicum annuum* varieties with susceptible and resistance cultivars. For accurate assessment of durable resistant germplasm in breeding program (Abang, 2003), pathogen diversity and geographical distribution information is

required. In pathotyping work, it is important to choose the appropriate isolates for screening of resistance in plant breeding programs. Previous studies on pathogenic variation among 7 cultivars susceptible *Capsicum annuum* and resistant cultivars of PBC932 *Capsicum chinense* and PBC80 and PBC81 on 10 isolates of *Colletotrichum acutatum* in Thailand revealed two pathotypes based on qualitative differences in infection of a reported resistant genotype (Than *et al*, 2008).

1.9 Objectives and aims of the study

A research programme was initiated in University of Malaya to investigate and report the *Colletotrichum* species that are found in Malaysia. This was due to the lack of such studies in Malaysia and the present study was to identify the species and pathotypes of *Colletotrichum* on different cultivars.

The main aims of this study are to identify the species of *Colletotrichum* isolated from infected chilli fruits of various farms in Peninsular Malaysia based on morphological features and molecular sequences and to investigate the pathotypes of *Colletotrichum* isolated from chilli and also from other fruits (mango, papaya and strawberry). The objectives and hypotheses that relate to the species identification section of the study are as follows: :

- To describe the morphology of *Colletotrichum spp* isolated from infected chilli, *Capsicum annuum* and *C. chinense.*

  H_o: different species of *Colletotrichum* have the same morphology

  H_1: different species have different morphology

- To describe the genetic variations of molecular sequences of *Colletotrichum* spp isolates based on ITS sequences.

  H_o: different species have the same genetic variations

  H_1: different species have different genetic variations

- To produce phylogenetic trees in order to confirm the *Colletotrichum* sp. and determine species relationships

  H_o: all species are equally related to one another

  H_1: some species are more closely related than others

In order to address the issues of pathotypes , the objectives are:

- To identify the pathotypes of *Colletotrichum acutatum*, *C. truncatum* and *C. gloeosporioides* based on the degree of infection due to inoculation bioassays of 3 varieties of chilli with different levels of ripeness mature and immature fruits.

  H_o: different chillis produce same pathotypes for each *Colletotrichum* species

  H_1: different chillis produce different pathotypes for each *Colletotrichum* species

- To identify the pathotypes based on the degree of infection isolated from papaya and strawberry on chilli pepper fruit.

  H_o: different host fruits produce same degree of infection and pathotypes.

  H_1: different host fruits produce different degree of infection and pathotypes.

Chapter 2 General materials and methods

2.1 Collection of chilli samples

Samplings were carried out throughout Peninsular Malaysia March 2008 until March 2009. Infected chilli were collected from the diseased plants from 22 farms of 9 states that represented 4 regions of Peninsular Malaysia; North (Perlis, Pulau Pinang and Kedah), Central (Selangor), South (Johore), East (Kelantan, Pahang and Terengganu. Hierarchical sampling was performed following the method by McDonald (1997), whereby within a farm a quadrat of 30x30 m was randomly selected to collect infected chilli samples. Five infected chilli fruits were collected from each of the five spots located at the four corners and the middle of the quadrat. The infected fruits were collected and kept in individual plastic bags and stored in ice to prevent them from cross contaminations and further rotting due to the weather and heat before being processed in the laboratory. Specimens were processed as soon as they are in the laboratory.

Severity of disease on farms was categorized as highly severe, moderate and non-severe. Severity were assessed by observation when walking throughout the farms, highly severe infection when every plants was infected and showed typical anthracnose with lesions, moderately severe when some plants appeared to be healthy without infections, while alternating with plants which had a few infected fruits and lastly non severe when infections were barely visible. Please refer to Table 2.1 for the list of farms visited, severity of disease, farms conditions and type of plantation.

2.2 Farms conditions

There were between two to three farms visited in each state. Farms visited were managed by participants under the project of 'Tanaman Kemajuan Pemakanan Malaysia', whereby the chilli cultivars planted by farmers were 'Kulai Kepala Besar', 'Cili Solok', 'MC 568' and other varieties of 'Kulai'. Farm conditions depended on the climatic condition, locality of the farms, watering system and planting method (Table 2.1). Humidity of the farm (dry or damp) was recorded as it depended on when the chilli was collected. Rainy season caused dampness in the farm or water soaking in the ground and depended on how the excess water is being channelled off. For types of watering systems such as drip tape, sprinkler and manual watering, all of which supplied different levels of humidity to the farms. Soil type determined the level of water holding capacity and the appropriate type of plants to crops planted in the ground soil. Some of the farms visited practiced rotation cropping to limit the spread of pathogens that had a wide range of host-diseases. For example, *Colletotrichum* spp. can cause anthracnose on a wide host of fruits such as papaya, strawberry, mango, citrus and chilli. Fungicides used extensively were Amistar, Score, Mancozeb, Topsin and Miller M-45. The application of fungicide depended on the weather and plant condition, while the frequency of application depended on the different conditions of the farm. Other insecticides were also applied to the plants to control insects, thrips and larvae diseases infestation.

Table2.1. List of farms details and conditions.

State	Farm	GPS	Host	Severity	Farms condition	Type of planting	Watering system
Perlis	Bukit Minit	N 06° 35.523' E 100° 14.358'	Kulai hybrid	Non severe	Damp	Ground	drip
Kedah	Pokok Sena	N 06° 29.136' E 100° 32.034'	Kulai	Non severe	Damp	Fertigation	drip
Penang	Desa Damai	N 05° 21.901' E 100° 26.399'	MC151 (kulai)	Non severe	Damp	Fertigation	drip
	Kebun Sireh	N 05° 21.591' E 100° 26.081'	Kulai	Non severe	Damp	Fertigation	drip
	Guar Perahu	N 05° 25.575' E 100° 28.727'	MC151,	Non severe	Damp	Fertigation	drip
			MC223, M568				
Perak	Gua Tempurung	N 04°23.889' E 101° 10.007'	Kulai	Severe	Dry	Fertigation	sprinkler
	Titi Gantung	N 04° 21.345' E 100°50.800'	Kulai king	Severe	Dry	Fertigation	drip
	Behrang	N 03°45.382' E 101°29.095'	Kulai	Moderate	Dry	Ground	drip
Selangor	Sri Keledang	N 03° 45.330' E 101° 19.906'	Kulai Kepala besar	Moderate	Dry	Ground	manual
Johor	Yong Peng, Johor	N 02° 03.276 'E 102°53.581	Ipoh hybrid	Moderate	Dry	Ground	sprinkler
	Segamat, Johor	N 02° 32'198' E 102° 55.024'	Kulai	Moderate	Dry	Ground	sprinkler
Pahang	Ulu Cheka, Jerantut	N 03°54.023' E 102° 11. 489’	Kulai Kepala besar	Severe	Dry	Ground	drip
	Janglau, Pahang	N 02°43.138' E 103° 33.907'	Kulai hybrid	Severe	Damp	Ground	sprinkler
	Pematang Lawang	N 03° 42.259' E 103° 18.575'	Kulai	Severe	Damp	Ground	sprinkler
	Pekan	N 03° 28.870' E 103° 21.659'	Kulai	Severe	Damp	Ground	sprinkler
	Merapoh, Kuala Lipis	N 04° 42.967' E 101° 59.937'	Kulai	Moderate	Damp	Ground	drip
Kelantan	Lojing	N 04° 37.253' E 101° 27.654'	Kulai hybrid	Moderate	Damp	Ground	sprinkler
	Kg Melawi, Bachok	N 06° 00.167' E 102° 24.585'	Kulai hybrid	Severe	Damp	Ground	drip
	Kampung Kuchelong	N 06° 01.179' E 102° 20.448'	Cili solok	Severe	Damp	Ground	drip
Terengganu	Besut	N 05° 39.199' E 102° 37.116'	Kulai	Severe	Damp	Ground	drip
	Marang	N 05° 04.505' E 103° 15.207'	Kulai hybrid	Non severe	Damp	Ground	sprinkler

Table 2.2. List of infected chilli collected, number of screened isolates and isolates being utilized in this study.

State	Farm	Infected chilli infected (n)	Screened isolates	Utilized
Perlis	Bukit Minit	20	16	1
Kedah	Pokok Sena	0	0	0
Penang	Desa Damai	7	5	0
	Kebun Sireh	16	11	1
	Guar Perahu	18	5	0
Perak	Gua Tempurung	15	10	0
	Titi Gantung	21	10	0
	Behrang	5	5	1
Selangor	Sri Keledang	12	10	0
Johor	Yong Peng, Johor	60	53	2
	Segamat, Johor	25	21	3
Pahang	Ulu Cheka, Jerantut	81	10	0
	Janglau, Pahang	119	21	2
	Pematang Lawang	50	10	0
	Pekan	48	15	1
	Merapoh, Kuala Lipis	24	7	0
Kelantan	Lojing	44	10	0
	Kg Melawi, Bachok	31	28	2
	Kampung Kuchelong	65	59	2
Terengganu	Besut	26	20	1
	Marang	14	10	0
Total		711	336	16

Figure 2.1 Anthracnose symptoms on *Capsicum annuum* in the field.

Farms under excess moisture normally had a high incidence of infection of anthracnose disease. This was observed in the farms in Jerantut, Janglau, Pematang Lawang, Pematang Pauh (Pahang), Kg. Melawi, Bachok, Kg Kuchelong (Kelantan) and Besut (Terengganu). Farms were damp due to flood or poor drainage management applied to the farm. This happened in states of Pahang, Kelantan and Terengganu due to monsoon season which flooded the farms. Thus, incidence of disease was extremely high and almost 100% of plants were infected. Poor management such as lack of drainage and farm hygiene occurred on the farms in Jerantut and Bachok.

In Perak, farms (Gua Tempurung and Titi Gantung) were planted using fertigation method and although the soils were generally dry, incidence of anthracnose was severe with almost all plants infected. Planting density was quite high with no gaps or interval between plants.

The incidence of disease on farm in Perlis (Bukit Minit), Kedah (Pokok Sena) and Penang (Desa Damai, Kebun Sireh and Guar Perahu) and Terengganu (Marang) was not high even though the farms were observed to be damp but with very good management and farm hygiene.

Farms in Perak (Behrang), Selangor (Sri Keledang) and Johor (Yong Peng and Segamat) was moderate. This was due to the farms being properly managed and the farms were basically well drained and dry. Please refer Table 3.1 on information of farms, GPS locality and farms condition.

Incidence of occurrence of infection of *Colletotrichum* species showed that in the farms that were damper, there were many infected fruits. Moisture levels are a key factor in the establishment of an epidemic in chilli farms by *Colletotrichum* spp. (Taylor, PWJT, personal communication). This can be seen at the sites such as Janglau and Jerantut and in the east coast where incidence of infection increased and almost entire farms were infected with *Colletotrichum* following floods, and monsoonal weather. Water irrigation also affects disease incidence of *Colletotrichum* in the farms. Over head sprinkler watering systems induce high humidity within the canopy of chilli plants which results in increased infections in the farms of Janglau, Pematang Lawang and Pematang Pauh (Pahang), Lojing (Kelantan) and Marang (Terengganu) Where water was delivered by a drip system there was lower infection rate such as in Bukit Minit, (Perlis), Pokok Sena (Kedah), Desa Damai, Kebun Sireh, Guar Perahu (Penang), Behrang (Perak), and Merapoh (Pahang).

In some farms such as Titi Gantung (Perak) there was almost 100% of infection due to poor management caused improper way of sanitizing the infected diseased plant away from the healthy plants, Farms in Jerantut (Pahang); Bachok and Kampung

Kuchelong (Kelantan) and Besut (Terengganu) also had very high infection due to flooding. (Refer Table 2.1 and 2.2 for information on farms condition and number isolates collected).

2.3 Culturing and screening of isolates

Preliminary screening of cultures was done to choose the isolates that will be utilized in this study. As stated in table 2.2, number of isolates collected, isolates screened (some isolates were tentatively identified up to species level by morphological characters, some isolates were contaminated and discard and unidentified isolates and not contaminated isolates were kept in vials) and isolates utilized for further studies were recorded.

Upon transferring the chilli samples into the laboratory, the fungi was cultured by cutting three 5 x 5 mm^2 pieces of the infected tissue of the fruits and the pieces were surface sterilized by dipping in 1% sodium hypochlorite for 3 min and rinsed with sterile distilled water three times. Tissue were then placed on fresh PDA plates and incubated at 28ºC for 7 days. The growing edges of fungal hyphae were then transferred to new plates containing fresh potato dextrose agar. Samples were cultured in laboratory on fresh PDA plates for incubation for 7 days. After 7 days, plates were preliminary screened by examining the conidia shapes of fungal species. Some samples were not screened due to the inconsistency of the samples or infection by other pathogens or mites, thus cultures were discarded. All isolates screened were transferred from petri dish and into vials and kept at room temperature.

2.4 Isolation

The isolates from the infected fruits were cultured on the potato dextrose agar for about 7 to 9 days and sub-culturing works were continued to produce more isolates for further

studies. Isolates of *Colletotrichum* were preserved using sterile distilled water in cryovials for long term storage (Harold & Dorworth, 1994). Isolates were grown in potato dextrose agar (Difco) and incubated for 5-7days at 28º*C*. Young mycelia were collected for future compilation by punching them using a cork borer to produce a plug of 6 mm. 8-10 plugs of mycelia were transferred into 2ml cryovials and stored under room temperature.

2.5 Single spore isolation

Single spore isolation was carried out using the 7-day cultures. These cultures were sub-cultured on fresh PDA plates for spore germination. Spores were monitored after 12 hours of incubation in nutrient agar. Spores were viewed through the dissecting microscope 40X, picked using sterile needle and cultured on a new agar plate.

2.6 Sampling and isolation from non-host of *Capsicum annuum*

Samples of *Colletotrichum* were isolated from strawberries were collected from two farms (Agro-technology Park, MARDI and KHM Strawberry and Jam) in Cameron Highlands, Pahang. Random sampling method was applied in obtaining the samples in the farm. Samples collected were plugged and kept in the small plastic bags with labels and stored in ice to prevent the fruits from rotting.

Samples of *Colletotrichum* were isolated from papayas that were bought from SS2, Petaling Jaya, Selangor. *Colletotrichum* species from these hosts were used for cross inoculation studies on *Capsicum annuum*.

Methods of culturing and screening of isolates, isolation and single spore isolation from both papaya and strawberry samples have the same methodology as follows. In the laboratory, infected tissue of the hosts were cut into three 5 x 5 mm^2 pieces of tissue and surface sterilized by dipping in 1% sodium hypochlorite for 3 min

and rinsed with sterile distilled water three times. Tissue were then placed on fresh PDA plates and incubated at 28°C for seven days. The edges of the growing fungal hyphae were then transferred to new plates containing fresh potato dextrose agar. Samples were cultured in laboratory on fresh PDA plates for incubation for 7 days. After 7 days, plates were preliminary screened by examining the conidia shapes of the fungi. Further methods on cross inoculation are explained in chapter 5.

Chapter 3 Morphological characteristics of *Colletotrichum* sp isolated from *Capsicum annuum* in Peninsular Malaysia

3.1 Introduction

Anthracnose of fruit and vegetables such as mango, papaya and chilli (*Capsicum* spp.) is often caused by a complex of *Colletotrichum* species which are difficult to differentiate at the species level based on culture characteristics (Than *et al*. 2008; Ranathunge *et al*., 2012). Malaysia has large tropical fruit and chilli industries which are at risk to infection by new exotic diseases entering Malaysia from SE Asia. There are over 100 species of *Colletotrichum* species that cause anthracnose disease (Cannon *et al*. 2012), and although many are common throughout the SE Asian region there are species and pathotype variants of species that are exotic to Malaysia.

Several major *Colletotrichum* spp. have already been isolated from these crops include the falcate-shaped spores of *C. truncatum* and the oblong-shaped spores of the *C. acutatum* and *C. gloeosporioides* complexes. Several species often infect a broad range of host species, co-exist in the one plant and in the same plant tissue making identification difficult. In chilli fruit, *C. truncatum*, *C. acutatum* and *C. gloeosporioides* have all been isolated from the fruit and have been shown to vary in their level of pathogenicity and ability to infect acrosss different host species (Than *et al*. 2008; Mongkolporn *et al*. 2010).

Traditionally, morphological characteristics based on colony colour, growth rate, spore size and shape; as well as host range have been used to identify *Colletotrichum* spp. Than *et al*, (2008) showed that spore morphology and colony growth rates on PDA at 25^{o}C differentiated the *Colletotrichum* species that caused anthracnose disease in chilli in Thailand into three distinct species – *C. acutatum, C. gloeosporioides* and *C.*

truncatum. *Colletotrichum acutatum* and *C. gloeosporioides* have always been difficult species to differentiate based on morphological characters (Cannon *et al*., 2012).

Denoyes & Baudry (1995) stated that conidial shape alone was a useful character to differentiate *Colletotrichum* species pathogenic on strawberry. According to Grahovac *et al*., (2012), *Colletotrichum* species were differentiated based on several morphological and ecological parameters. The application of morphological characters such as colony colour, size and shape of conidia, presence and absence of setae and teleomorph and cultural characters (Sutton, 1980; Van der Aa *et al*., 1990; Gunnell & Gubler, 1992; Liyange *et al*., 1992; Sutton, 1992; Agrios, 2000; Smith & Black, 1990) have been key identification tools for *Colletotrichum* species such as *C. acutatum*, *C. truncatum* and *C. gloeosporioides*.

However, more recent studies have shown that morphological characters for identification *Colletotrichum* species were too variable and taxonomically uninformative (Crouch, 2009; Cai *et al*., 2009). The aim of this study was to identify the *Colletotrichum* species collected in Malaysia using growth rates, colony morphology and conidia morphology.

3.2 Materials and methods

3.2.1 Morphological study

Isolates were produced following the methods mentioned in Chapter 2. 16 isolates chosen for morphological study are listed in Table 3.1 and were sub-cultured**.** Morphological characteristics were categorized into colony and conidia morphology. Distinct morphological characters on PDA were observed in each morphology group after 7 days from sub-culturing. These isolates were grouped according to their colony

characteristics.

Petri dishes containing half strength potato dextrose agar (PDA) were inoculated in the centre of the plate with mycelia discs taken from the outer edge of the growing mycelium. Cultures were grown in darkness at 28ºC. Colony diameter was measured using ruler daily for one week with 3 replicates.

Slides of conidial spores were prepared with Lactophenol blue stain. Slides were examined using compound microscope Leica DM1000 LED. Conidia shapes were recorded and forty randomly chosen conidia for each isolates were measured using Leica Software using the same microscope. The ratio of length/width of conidia was calculated. Data were analysed with statistical analysis Statistica 8 software (Statsoft, UK) (Taylor, 2007)

3.3 Results

3.3.1 Morphological characteristics of isolates of *Colletotrichum* spp.

3.3.1.1 Colony morphology

From a total of 336 isolates that were screened, only 16 isolates were utilized for the morphology and growth rate study. Among the 16 isolates, five isolates were tentatively identified as *C. acutatum*, five isolates were tentatively identified as *C. gloeosporioides* and six isolates were tentatively identified as *C. truncatum*.

Among the *C. acutatum* isolates (Fig. 3.1), isolates A13, A15, CO4, E15 and F59 have fusiform shaped conidia. Isolates A13 and A15 had culture characters of white colony with thin aerial mycelia with mass of orange conidia on conidiomata. Isolate CO4 had white colony with dark flecking on the reserve, isolate E15 had grey with thin cottony mycelia and isolate F59 was greyish white cottony mycelia with masses of orange conidia on the conidiomata with fusiform shaped conidia.

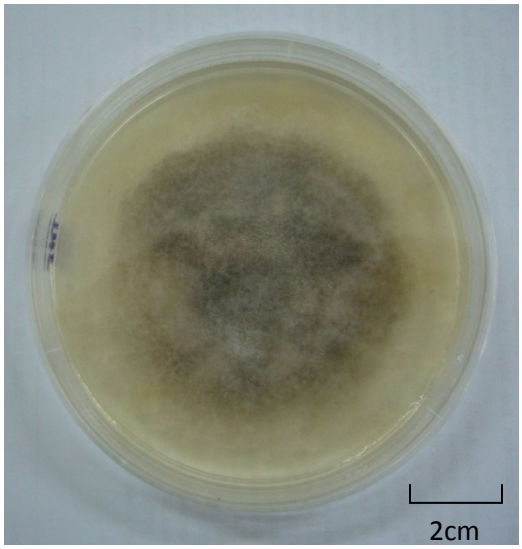
C. gloeosporioides isolates (Fig. 3.2) were represented isolates BKM, M01, N01, O05 and O11 have cylindrical conidia. All *C. gloeosporioides* isolates in this study had the same colony morphology; white thick cottony mycelia with orange conidia masses with cylindrical shaped conidia.

C. truncatum isolates (Fig. 3.3) comprised of isolates BO4, B16, DO6, EO2, F37 and OO3 have falcate shaped conidia. All *C. truncatum* isolates in this study had greyish white mycelia with black acervuli with orange conidia masses with falcate shaped conidia.

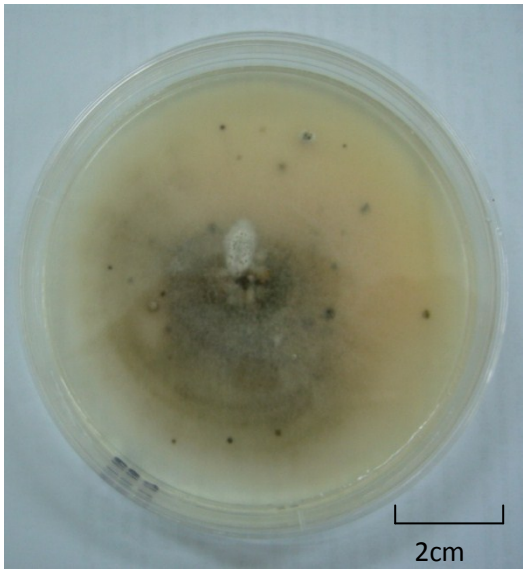
Table 3.1. Description of cultural characteristics and conidia morphology of *Colletotrichum* isolated obtained from chilli.

Farms/Market	Isolate	Cultural characteristics	Conidial morphology	Tentative identified species
Janglau, Pahang	A13	White colony with thin aerial mycelia with mass of orange conidia on conidiamata.	fusiform	*C. acutatum*
Janglau, Pahang	A15	White colony with thin aerial mycelia with mass of orange conidia on conidiamata.	fusiform	*C. acutatum*
Pekan, Pahang	C04	White colony with dark flecking on the reverse.	fusiform	*C. acutatum*
Yong Peng, Johor	E15	Grey with thin cottony mycelia.	fusiform	*C. acutatum*
Kampung Kuchelong, Kelantan	F59	Greyish white colony with slightly thin cottony mycelia.	fusiform	*C. acutatum*
Bukit Minit, Perlis	BKM	White thick cottony mycelia with orange conidia masses	cylindrical	*C. gloeosporioides*
Behrang, Selangor	M01	White thick cottony mycelia with orange conidia masses	cylindrical	*C. gloeosporioides*
Besut, Kelantan	N01	White thick cottony mycelia with orange conidia masses	cylindrical	*C. gloeosporioides*
Segamat, Johor	O05	White thick cottony mycelia with orange conidia masses	cylindrical	*C. gloeosporioides*
Segamat, Johor	O11	White thick cottony mycelia with orange conidia masses	cylindrical	*C. gloeosporioides*
Bachok, Kelantan	B04	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*
Bachok, Kelantan	B16	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*
Kebun Sireh, Pulau Pinang	D06	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*
Yong Peng, Johor	E02	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*
Kampung Kuchelong, Kelantan	F37	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*
Segamat, Johor	O03	Greyish white mycelia with black acervuli with orange conidia masses	falcate	*C. truncatum*

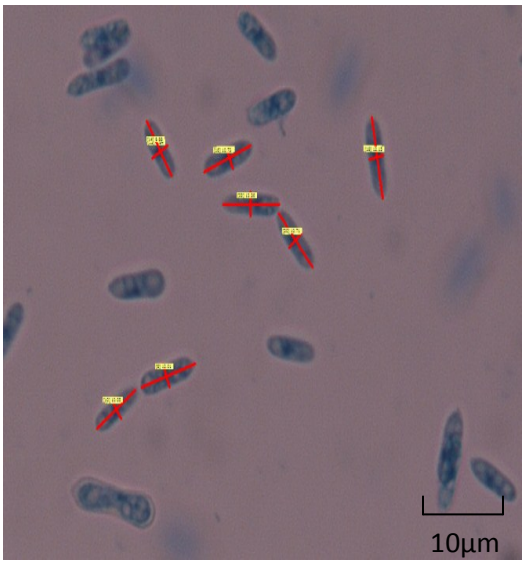
A. White colony with thin aerial mycelia with mass of orange conidia on conidiamata.



B. Grey with thin cottony mycelia

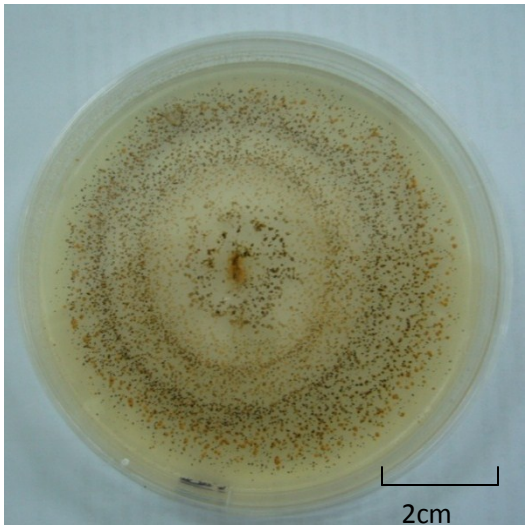


C. Grey whitish mycelia with dark flecking on the reverse

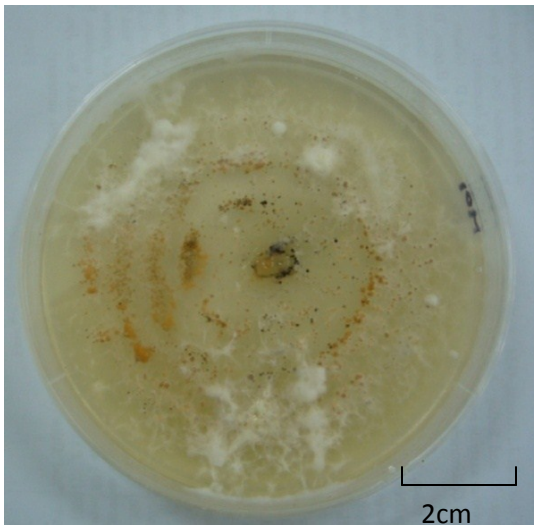


D. Fusiform shaped conidia

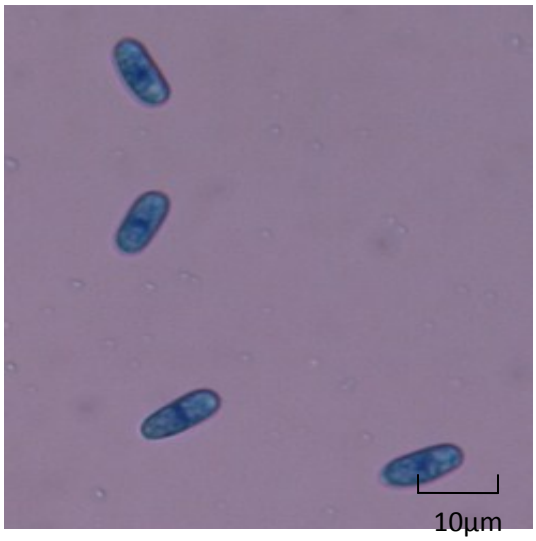
Figure 3.1. Colony characters and conidia for tentative identification of *Colletotrichum acutatum.*



A. Sporulating conidiomata with orange conidia mass.

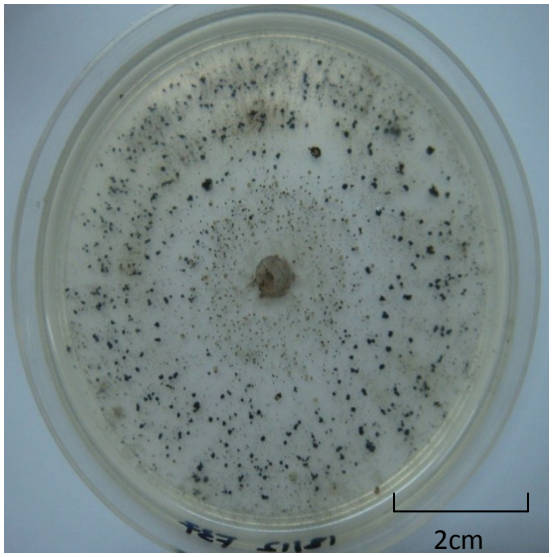


B. White thick cottony mycelia with orange conidia mass

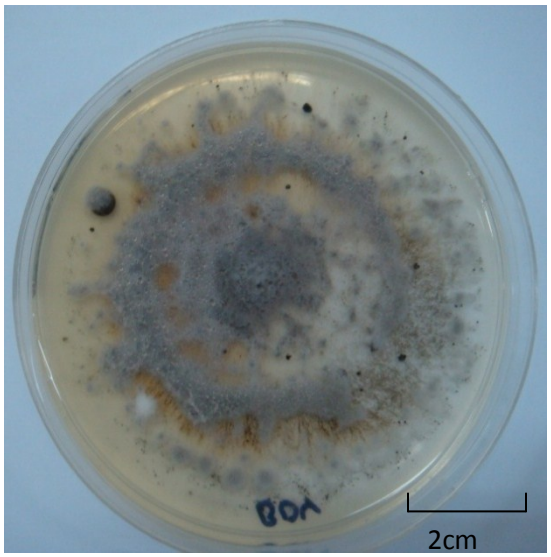


C. Cylindrical shaped conidia

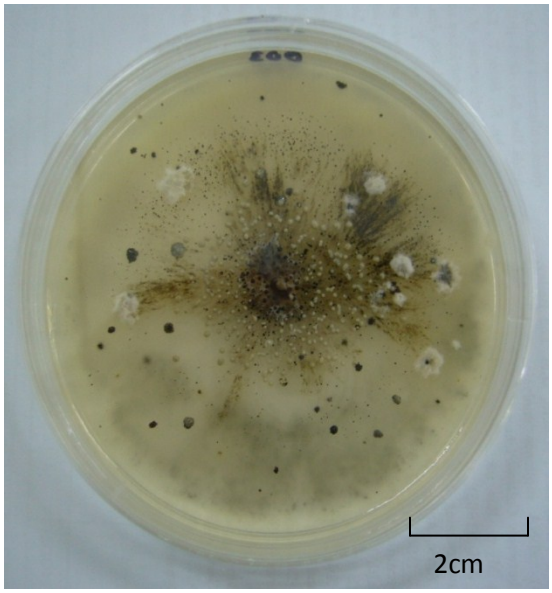
Figure 3.2. Colony characters and conidia for tentative identification of *Colletotrichum gloeosporioides.*



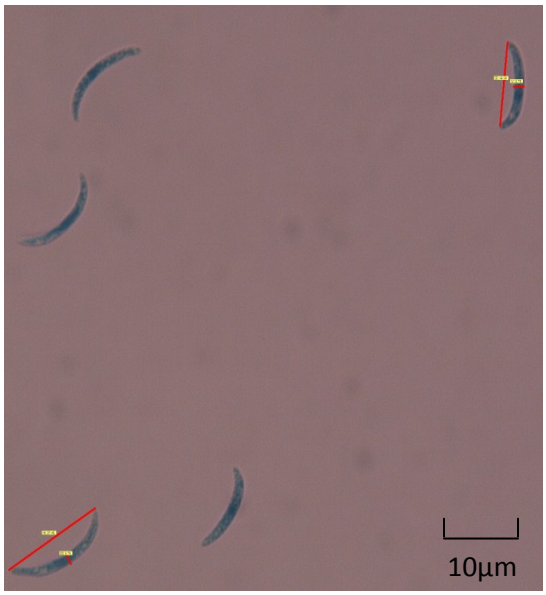
A. Greyish white mycelia with black acervuli with orange conidia mass



B. Greyish white mycelia with black acervuli with orange conidia mass



C. Greyish white mycelia with black acervuli with orange conidia mass



D. Falcate shaped conidia

Figure 3.3. Colony characters and conidia for tentative identification of *Colletotrichum truncatum.*

3.3.2 Growth rate analysis

There were significant ($p<0.05$) differences in growth rate (colony diameter/ day) between the three species of *Colletotrichum* with *C. gloeosporioides* having the fastest growth rate and *C. acutatum* the slowest growth (Table 3.2 and Appendix 2.0).

Among *C. acutatum* isolates, A13 had the fastest growth rate at 10.12 mm/ day. Isolates A15, CO4, E15 and F59 had average growth ranging from 5.54 mm/day to 5.95 mm/day.

Among *C. gloeosporioides* isolates, MO1, NO1 and O11 had the highest growth of 11.43 mm/ day while OO5 had the slowest growth with only 10.12 mm/ day.

Among *C. truncatum* isolates, BO4 had fastest growth rate of 9.02 mm/day. EO2 had the slowest growth with 6.29 mm/day. The growth rate for B16, DO6, F37 and OO3 was in the range of 6.86 mm/day to 8.33 mm/day.

Table 3.2. Growth rate of *C. acutatum*, *C. gloeosporioides* and *C. truncatum*

Species	*C. acutatum* (n=30)	*C. gloeosporioides* (n=30)	*C. truncatum* (n=36)	LSD
Mean growth rate (mm/day)	6.63^{a}	11.12^{b}	7.67^{c}	0.003152

Homogenous groups indicating no significant difference ($p>0.05$) by Fisher LSD test are shown by similar superscript alphabets.

Within all species, there were significant differences in growth rate between the isolates (Table 3.3). *C. acutatum* isolates, A13 and A15 showed variability in growth rates with isolate A13 having growth rate almost twice that of the other isolates in this species. *C. truncatum* isolate BO4 had significantly higher growth rate than the other isolates within the species, while EO2 had the slowest. There was no significant difference in growth rates between isolates of *C. gloeosporioides* except for OO5 which had significantly lowest growth rate. Please refer appendix 3.0 for the analysis.

Table 3.3. Mean growth rate of isolates within the three *Colletotrichum* species.

C. acutatum	A13	A15	CO4	E15	F59		LSD
Mean ± S. D	10.11±2.07[a]	5.55±0.49[b]	5.95±0.20[b]	5.79±0.20[b]	5.74±0.06[b]		0.932169
C. gloeosporioides	BKM	MO1	NO1	OO5	O11		LSD
Mean	11.19±0.20[a,b]	11.43±0.00[a]	11.43±0.00[a]	10.12±2.07[b]	11.43±0.00[a]		1
C. truncatum	BO4	B16	DO6	EO2	F37	OO3	LSD
Mean	9.02±0.31[a]	6.85±0.42[d]	8.24±0.44[b]	6.29±0.13[e]	7.29±0.24[c]	8.33±0.44[b]	0.640299

Homogenous groups indicating no significant difference ($p>0.05$) by Fisher LSD test are shown by similar superscript alphabets.

3.3.3 Conidia analysis

A total of 15 isolates were utilized in this analysis as isolate DO6 were not included due to poor sporulation. Conidia lengths of all species are significantly different. *C. truncatum* has longest conidia (mean=22.40µm) followed by *C. gloeosporioides* (mean=13.79µm) and *C. acutatum* (mean=9.72µm). However in terms of conidia width, there were no significant differences between *C. acutatum* and *C. truncatum* but *C. gloeosporioides* is significantly wider than both *C. acutatum* and *C. truncatum*.

C. truncatum had a more characteristic longer falcate shaped conidia compared to *C. acutatum* and *C. gloeosporioides.* The length and width of the spores were significantly different between the species of *Colletotrichum* ($p < 0.05$) with the sizes for *C. gloeosporioides* being significantly larger than for *C. acutatum* (Table 3.4).

Table 3.4. Conidia length and width for the three *Colletotrichum* species

	C. acutatum (n=200)	*C. gloeosporioides* (n=200)	*C. truncatum* (n=200)	LSD
Mean length (µm)	9.72[c]	13.79[b]	22.40[a]	0
Mean width (µm)	3.06[b]	3.88[a]	3.14[b]	0.301372

Homogenous groups indicating no significant difference ($p>0.05$) by Fisher LSD test are shown by similar superscript alphabets.

There were significant differences in spore size between isolates within each species (Table 3.5). Isolates A13, A15 and E15 showed almost the same length with 9.27, 9.33 and 9.41 µm. Width of the conidia also showed some differences between isolates with isolates A13, CO4 and F59 having the smallest spore width.

In *C. truncatum*, isolate B16, (23.72 µm) had the longest conidia compared to F37, (20.61 µm) which had the shortest (Table 3.5). Width of the conidia showed no

significant difference between isolates except for isolate F37 which had the largest spore width. Isolate DO6 was not included in this analysis due to poor sporulation of the culture.

Isolates of *C. gloeosporioides* showed intermediate size of conidia compared to the length of the other two species (Table 3.5). Isolate BKM had the shortest length of 11.99 µm and isolate O11 was the most significant longest (14.64 µm) while O11 was the narrowest of 3.51 µm.

Table 3.5. Mean conidia length and width between isolates of the three *Colletotrichum* species.

C. acutatum	A13 (n=40)	A15 (n=40)	CO4 (n=40)	E15 (n=40)	F59 (n=40)	LSD
Length mean (µm)	9.27^{c}	9.33^{c}	10.63^{a}	9.41^{c}	9.99^{b}	0.80217
Width mean (µm)	2.93^{b}	3.46^{a}	2.59^{c}	3.48^{a}	2.86^{b}	0.77794
C. gloeosporioides	BKM (n=40)	MO1 (n=40)	NO1 (n=40)	OO5 (n=40)	O11 (n=40)	
Length mean (µm)	11.99^{c}	14.10^{b}	14.19^{b}	13.99^{b}	14.64^{a}	0.69599
Width mean (µm)	4.66^{a}	$3.66^{b,c}$	$3.67^{b,c}$	3.89^{b}	3.51^{c}	0.91764
C. truncatum	BO4 (n=40)	B16 (n=40)	EO2 (n=40)	F37 (n=40)	OO3 (n=40)	
Length mean (µm)	$21.36^{b,c}$	24.48^{a}	23.72^{a}	20.61^{d}	21.82^{b}	0.25545
Width mean (µm)	3.11^{b}	2.88^{b}	3.02^{b}	3.93^{a}	2.76^{b}	0.67627

Homogenous groups indicating no significant difference ($p>0.05$) by Fisher LSD test are shown by similar superscript alphabets.

3.4 Discussion

Isolates of the same species from different localities had variation in colony morphology, especially for *C. acutatum*. Growth rate is one of the main comparative characters of colony in culture. *C. gloeosporioides* was able to be differentiated from C. acutatum because of a faster growth, while *C. truncatum* had a growth rate intermediate

to the other two species. According to Than *et al*. (2008) there were no significant difference in growth rates among the isolates within the same *Colletotrichum* species, but statistical difference was observed in the growth rate between the three different *Colleotrichum* species. The growth rate of *C. gloeosporioides* (11.12 mm day^{-1}), *C. truncatum* (7.61 mm day^{-1}) and *C. acutatum* (6.63 mm day^{-1}), were similar to the growth rates of the same species that infected chilli in Thailand (Than *et al*., 2008). Conidial morphology can be applied as criteria for delineation of *C. acutatum* from *C. gloeosporioides.*

The conidia shape and length was distinctive for *C. gloeosporioides*, *C. acutatum* and *C. truncatum*. The conidia for *C. truncatum* were slightly larger than those of the same species in the study of isolates from chilli (Than *et al*., 2008) and the type isolate (Damm *et al*., 2009) however natural variation is spore size would account for these mean differences (Table 3.5). Although the shape of the conidia for *C. acutatum* were fusiform and *C. gloeosporioides* cylindrical, there is generally large variation in shape and size that makes this character unreliable for separating these closely related species. The *C. acutatum* isolates were smaller compared to those in other studies especially compared to the size of spores of isolates from chilli in Thailand which were larger (Table 3.5). The size of the spores of *C. gloeosporioides* (13.8 µm) were similar to those in the study by Than *et al*; (2008) however, the size of the type species isolates reported by Weir *et al*. (2012) were much larger (16.7 µm; Table 3.5).

The variation in spore size of *C. acutatum* and *C. gloeosporioides* between studies may have been due to the influence of the different media and conditions used in the studies to culture the isolates; and the fact that type isolates may have originated from different hosts. More importantly there may be several different species within the Malaysian isolates that were not clearly differentiated by spore size alone. Further

studies using molecular analysis of fungal genes are required to validate taxonomic differences.

Table 3.6. Summary of conidia length (µm) and width (µm) among different studies

	C. acutatum (length x width)	***C. gloeosporioides*** (length x width	***C. truncatum*** (length x width
Khor (Current study)	9.72 ± 1.17 x 3.06 ± 0.46	13.79 ± 1.34 x 3.88 ± 0.73	22.39 ± 2.32 x 3.13 ± 0.99
Than *et al*., (2008)	13.5 x 3.5	13-14 x 3.5	21 x 3
Damm *et al*., (2009)			21.8 ± 1.9 × 3.8 ± 0.3
Weir *et al*., (2012)		16.74 × 5.1*	
Damm *et al*., (2012)	10.3 ± 1.9 × 3.4 ± 0.5		

*Average spore size across all species within the *C. gloeosporioides* complex.

Chapter 4.0 Molecular identification and phylogeny of *Colletotrichum* spp as estimated by ITS sequences

4.1 Introduction

The previous chapter identified Malaysian isolates of *Colletotrichum* spp from infected chilli fruit based on morphological characteristics. These were classified tentatively as the *Colletotrichum acutatum, gloeosporioides;* and *truncatum* species. However, recent taxonomic studies using multi gene phylogeny by Weir *et al*., (2012) and Cannon *et al*., (2012) have showed that the *C. acutatum* and *C. gloeosporioides* may represented as distinct complexes composed of many new *Colletotrichum* species. Fungi isolates from infected chilli fruit were separated into *C. siamense* believed to belong in (‘*gloeosporioides*’ complex); and *C. scovillei* and *C. brisbenense* believed to belong in (‘*acutatum*’ complex). A problem with this new taxonomy was that the new species were based only on a few isolates primarily originating from Indonesia and Thailand. There has not been any systematic study of the taxonomy of *Colletotrichum* species infecting chilli in Malaysia based on the new taxonomy.

Multigene phylogeny studies of fungi are usually based on 3 to 6 fungal gene sequences such as ITS, 18S, LSU, SSU and etc. Nevertheless, the ITS gene sequence alone has been shown to differentiate many *Colletotrichum* species (Cannon *et al*., 2012) and was selected as the universal ‘barcoding’ gene to categorize all fungi (Schoch *et al*., 2012). In this chapter, molecular sequence data from the ITS gene was studied to confirm the identification as well as to infer the phylogenetic relationships for isolates of *Colletotrichum* collected in Malaysia.

4.2 Materials and Methods

4.2.1 *Colletotrichum* isolates

A total of 18 isolates were used for the molecular identification of phylogenetic study out of 19 isolates. Among the 18 isolates, BO4 was not included due to the failure of PCR amplification. 16 isolates were A13, A15, CO4, E15, F59 (tentatively identified as *C. acutatum*), BKM, MO1, NO1, OO5, O11 (tentatively identified as *C. gloeosporioides*), B16, DO6, EO2, F37 and OO3 (tentatively identified as *C. truncatum*), whereas an additional 3 isolates were obtained from papaya (P2P1D) and strawberry host (STP22, TH5). Isolates P2P1D was tentatively identified as *C. gloeosporioides*. Isolates STP22 and TH5 were tentatively identified as *C. acutatum*.

4.2.2 DNA extraction

Mycelia were harvested and extracted from cultures growing on PDA at 28^{o}C using DNeasy Plant Mini Kit by Qiagen. Fungal mycelia were ground to powder with liquid nitrogen and transferred into 2 ml tubes for evaporation by centrifuging for 30 sec at full speed. 400 µl of Buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml) were added to 20 mg mycelia and vortexed vigorously. Conspicuous mycelia clumps were removed by pipetting or vortexing. Lysate were incubated for 10 min at 65°C while inverting the tube during incubation. 130 µl of Buffer AP2 were added to the lysate and incubated for 5 minutes in ice. Lysate were centrifuged at 14000 rpm for 5 minutes. The supernatant were placed in a QIAshredder Mini spin column for the shearing of DNA. The lysate produced in the QIAshredder Mini spin column was pipetted to 2 ml collection tubes and centrifuged at 14000 rpm for 2 min. The flow through fraction was transferred into a new tube without disturbing the cell-debris pellet. 450 µl of lysate was recovered although some mycelia produced less lysate. 675

µl of Buffer AP3/E was added to the 450 µl of lysate, or the amount of Buffer AP3/E was adjusted according to the volume of the lysate. It was crucial that Buffer AP3/E was pipetted directly into the cleared lysate and mixed immediately. 650 µl of mixture including the precipitation which formed in the DNeasy Mini spin column was pipette into 2 ml collection tubes, centrifuged at 8000 rpm for 1 minute with the flow through being discarded. This procedure was repeated with the remaining samples in the same collection tubes and discard along with the flow through in completion of the procedure. DNA extracted was visualized using 1.3% TBE agarose gel and electrophoresis solution.

4.2.3 PCR amplification of ITS region

PCR was performed in 50 µl reactions comprising 1 µl each of 0.5 µM forward and reverse primers, 25 µl 2x DyNazyme II master mix (Finnzymes) and 20 µl of distilled H_20. PCR was performed in a Bio-Rad MyCycler^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc.) using primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990)with denaturation of 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 60-61.5°C for 1 minute and elongation at 72°C for 1 minute, with a final extension step of 72°C for 7 minutes. PCR products were verified by staining with ethidium bromide on 1.3% agarose electrophoresis gels. PCR products were sent for purification and sequencing by Next Gene Scientific (Malaysia).

4.2.4 Basic Local Alignment Search Tool (BLAST)

Chromatograms of the forward and reverse sequence reads were checked, edited and contig sequences were assembled for each isolate using Bioedit program. ITS sequences were submitted to BLAST (Altschul *et al*., 1997) to search for identical and

similar sequences from the GenBank database. The 3 most significant alignments (ranked by scores) from Mega BLAST were cross-checked with morphological description for microfungi identification. Please refer Table 4.1 for list of reference sequences obtained from BLAST searches.

4.2.5 Alignment of DNA sequence

Although BLAST searches were done, the result sequences were not used for phylogenetic analysis because their identification may not have been accurate and were unreliable. Thus, the reference sequence data were obtained from published journals (Table 4.2). Multiple sequence alignment of ITS sequences of isolates was performed with the list of verified reference sequences (Table 4.2) based on voucher specimens or epitypes from published journal using CLUSTAL X program.

Alignments were inspected and confirmed visually for any ambiguous alignments which were then corrected manually. Initially, multiple sequence alignment was carried out according to alignment parameters of pairwise alignment and multiple alignments with the value of gap opening penalty of 10.00 and gap extension penalty of 0.10. Nucleotide frequencies of each isolate used in the alignment were tabulated by MEGA 4.0. Alignment files (.mas) for ITS were exported to (.meg) format and input into DnaSP 4.0 (Rozas *et al*., 2003) to analyse and identify the amount of position invariable sites and parsimony informative sites. The alignment files were then exported for analysis in PAUP 4.0b (Swofford, 2002) in Nexus format (.nex). Please refer Appendix 4.0 for the alignment of the ITS region sequences.

4.2.6 jModeltest analysis

The best evolutionary model was chosen for the ITS sequence alignment using jModeltest 0.1.1 (Posada, 2008). Please refer Appendix 5.0 for modeltest result. The

parameters of the best evolutionary model selected was incorporated into Neighbour Joining and Bayesian analyses. NJ tree was estimated using PAUP, whereas MrBayes v3.1.2 was used to build a Bayesian tree. Parsimony tree do not require any evolutionary model for construction, and was constructed using PAUP. Bootstrapping if 1000 replicates was employed.

4.2.7 Bayesian analysis of phylogeny

Bayesian analysis aims to find the most likely tree by sampling a subset of trees that have the highest posterior probability (Huelsenbeck *et al*., 2001), calculated by integrating the prior probability of parameters with the likelihood of our tree. The random sampling of our parameters will give a large set of trees with different posterior probability values. In order to sample trees with only the highest posterior probability, Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) was used from 4 chains of 1,000,000 generations. All other parameters were left at default. A total of 1001 trees were sampled from the posterior probability distribution (one in every 100 generation) and the initial 25% trees were discarded as 'burn-in' to ensure the chains have reached stationarity. A summary of the workflow is shown in Fig. 4.1.

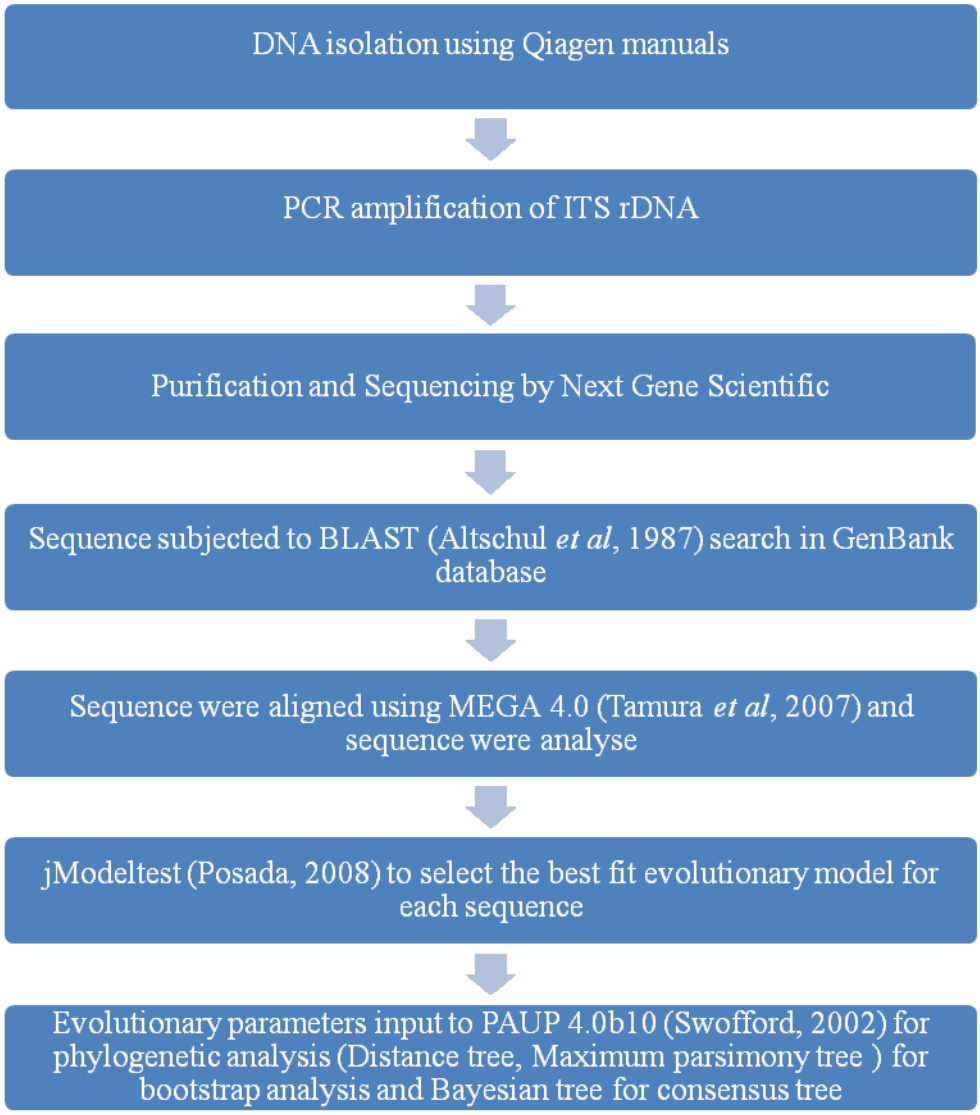


Figure 4.1. Flowchart showing the workflow of isolating, identifying and inferring phylogenetic relationships using PCR sequencing of the nuclear ITS rDNA region.

4.3 Result

4.3.1 Sequence alignment of ITS region characteristic

The ITS region for all 18 isolates was successfully amplified by PCR. The size of the entire region ranged from 500-600 base pair. The 3 most significant alignments (ranked by score) from MegaBLAST for ITS sequence were used in combination with the morphological descriptions of all 18 isolates to find the consensus identity for the microfungi (Table 4.1). The results of the BLAST comparison helped to resolve the uncertainties of several identities. However, the BLAST comparison was insufficient to confer precise species name, with some identified to the genus level only.

ITS alignment consisted of 31 taxa, inclusive of 18 isolates of *Colletotrichum* and 13 reference sequences. The total alignment length was 496 bp or 496 sites. From the analysis of sequence alignment, 123 were conserved sites, 371 were variable sites, including singtон and parsimony informative totalled 99 sites.

Table 4.1. Identification of *Colletotrichum* species based on the MegaBLAST comparison of the ITS1-5.8S-ITS2 data as well as the morphological characteristics. The 3 most significant alignments showed highest scores of 98-100% identity matches.

Isolates	GenBank most significant sequences	Comparison of cultural characteristics
A13	Accession: DQ300347.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum sp. ID03	2. Sparse aerial mycelia
	Accession: AJ301921.1	3. Tentatively identified as *C. acutatum*
	Colletotrichum acutatum strain BBA70349	
	Accession: AY266405.1	
	Glomerella acutata strain G2	
A15	Accession: AJ301921.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum acutatum strain BBA70349	2. Sparse aerial mycelia
	Accession: DQ300347.1	3. Tentatively identified as *C. acutatum*
	Colletotrichum sp. ID03	
	Accession: AJ301920.1	
	Colletotrichum acutatum strain BBA70348	
B16	Accession: JQ936134.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum lupini strain M2P28E	2. Diurnal zonation of dense mycelia
	Accession: JN121206.1	3. Tentatively identified as *C. truncatum*
	Colletotrichum simmondsii strain 1572	
	Accession: AY266405.1	
	Glomerella acutata strain G2	

Table 4.1, continued		
BKM	Accession: HM211224.1	1. Isolates showed to produced colonies with orange colour dense mycelia
	Fungal sp. GZ-2010a isolate PY37	2. Tentatively identified as *C. gloeosporioides*
	Accession: JX624297.1	
	Colletotrichum sp. JK74	
	Accession: KC207404.1	
	Colletotrichum gloeosporioides strain hjh1	
CO4	Accession: DQ300347.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum sp. ID03	2. Sparse aerial mycelia
	Accession: AJ301920.1	3. Tentatively identified as *C. acutatum*
	Colletotrichum acutatum strain BBA70348	
	Accession: DQ300349.1	
	Colletotrichum sp. SM01	
DO6	Accession: KC460308.1	1. Dense mycelia
	Colletotrichum truncatum strain tc-1	3. Tentatively identified as *C. truncatum*
	Accession: GQ369594.2	
	Colletotrichum capsici strain 10858	
	Accession: JQ936210.1	
	Colletotrichum capsici strain C3P12C	

Table 4.1, continued		
EO2	Accession: JX971163.1	1. Isolates showed sparse white or grey mycelia with conidiomata with
	Colletotrichum truncatum strain CTM40	beige spore masses
	Accession: JX971162.1	3. Tentatively identified as *C. truncatum*
	Colletotrichum truncatum strain CTM39	
	Accession: JX258749.1	
	Colletotrichum truncatum strain C2P1B2	
E15	Accession: AJ301921.1	1. Isolates showed sparse aerial mycelia
	Colletotrichum acutatum strain BBA70349	3. Tentatively identified as *C. acutatum*
	Accession: AJ301920.1	
	Colletotrichum acutatum strain BBA70348	
	Accession: DQ300347.1	
	Colletotrichum sp. ID03	
F37	Accession: JX971163.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum truncatum strain CTM40	2. Diurnal zonation of dense mycelia
	Accession: JX258749.1	3. Tentatively identified as *C. truncatum*
	Colletotrichum truncatum strain C2P1B2	
	Accession: JX258714.1	
	Colletotrichum boninens e strain C2P4F5	

Table 4.1, continued		
F59	Accession:JN715841.1	1. Isolates showed sparse aerial mycelia
	Colletotrichum sp. Q030	3. Tentatively identified as *C. acutatum*
	Accession: AM991131	
	Glomerella acutata isolate PT811	
	Accession: EF221832	
	Glomerella acutata isolate CIAD/GAQ-03	
MO1	Accession: HM211224.1	1. Isolates showed to produced colonies with orange colour dense mycelia
	Fungal sp. GZ-2010a isolate PY37	in the center and pale yellow towards the margin of the zonation
	Accession: AY266378.1	2. Tentatively identified as *C. gloeosporioides*
	Colletotrichum gloeosporioides strain CMUBE1814	
	Accession: HM537077.1	
	Fungal endophyte sp. g110	
NO1	Accession: AY266378.1	1. Isolates showed to produced colonies with orange colour dense mycelia
	Colletotrichum gloeosporioides strain CMUBE1814	in the center and pale yellow towards the margin of the zonation
	Accession: GU174549	2. Tentatively identified as *C. gloeosporioides*
	Colletotrichum gloeosporioides culture-collection ICMP:17785	
	Accession: JX010258.1	
	Colletotrichum siamense strain C1316.6	

Table 4.1, continued		
OO3	Accession: KC460308.1	1. Isolates showed conidia with masses of orange conidia
	Colletotrichum truncatum strain tc-1	2. Diurnal zonation of dense mycelia
	Accession: JX258748.1	3. Tentatively identified as *C. truncatum*
	Colletotrichum truncatum strain M2P3D6	
	Accession: JX258714.1	
	Colletotrichum boninense strain C2P4F5	
OO5	Accession: JX258803.1	1. Isolates showed to produced colonies with orange colour dense mycelia
	Colletotrichum gloeosporioides strain M96	2. Tentatively identified as *C. gloeosporioides*
	Accession: FJ455526.1	
	Colletotrichum gloeosporioides strain HT72	
	Accession: KC507276.1	
	Colletotrichum sp. FT2	
O11	Accession: EF423519.1	1. Isolates showed to produced colonies with orange colour dense mycelia
	Glomerella cingulata isolate P013	2. Tentatively identified as *C. gloeosporioides*
	Accession: JX010159.1	
	Colletotrichum siamense strain C1254.6	
	Accession: JQ936124.1	
	Colletotrichum gloeosporioides strain M63	

Table 4.1, continued		
P2P1D	Accession: AJ301979	1. Isolate showed colonies with greyish white to dark grey dense mycelia.
	Colletotrichum acutatum strain BBA71367	2. Tentatively identified as *C. gloeosporioides*
	Accession: EF423535	
	Glomerella cingulata isolate P060	
	Accession: AB042319	
	Glomerella cingulata MAFF 305974	
STP22	Accession: AJ301921	1. Isolates showed white or pale grey colonies of diurnal zonation.
	Colletotrichum acutatum strain BBA70349	3. Tentatively identified as *C. acutatum*
	Accession: AJ301975	
	Colletotrichum lupini	
	Accession: FJ972601	
	Glomerella acutata (anamorph: *Colletotrichum acutatum*)	
TH5	Accession: GU183317	1. Isolates showed conidia with masses of orange conidia with white or
	Colletotrichum simmondsii culture-collection BRIP:4704	plae grey colonies of diurnal zonation.
	Accession: AJ301982	3. Tentatively identified as *C. acutatum*
	Colletotrichum acutatum strain BBA71371	
	Accession: EF221832	
	Glomerella acutata isolate CIAD/GAQ-03	

Table 4.2. Reference sequences utilized in this study for the construction of the phylogenetic trees.

Accession	Reference description	Journal
GU227862	*Colletotrichum truncatum culture-collection CBS:151.35*	Damm *et al*.(2009)
FJ972612	*Colletotrichum asianum isolate BPDI4*	Prihastuti *et al*. (2009)
JX010276	*Colletotrichum queenslandicum strain ICMP 1778*	Weir *et al*. (2012)
JX010171	*Colletotrichum siamense strain C1315.2*	Weir *et al*. (2012)
JX010173	*Colletotrichum fructicola strain C1316.21*	Weir *et al*. (2012)
JX010176	*Colletotrichum aeschynomenes strain 3-1-3*	Weir *et al*. (2012)
EU371022	*Colletotrichum gloeosporioides voucher IMI 356878*	Cannon *et al*. (2008)
JQ948267	*Colletotrichum scovillei culture-collection CBS:126529*	Damm *et al*. (2012)
JQ948276	*Colletotrichum simmondsii culture-collection CBS:122122*	Damm *et al*. (2012)
JQ948291	*Colletotrichum brisbanense culture-collection CBS:292.67*	Damm *et al*. (2012)
JQ948288	*Colletotrichum indonesiense culture-collection CBS:127551*	Damm *et al*. (2012)
JQ005776	*Colletotrichum acutatum culture-collection CBS:112996*	Damm *et al*. (2012)
GQ485607	*Colletotrichum cliviae strain CSSK4*	Yang *et al*. (2009)

4.3.2 Evolutionary model selection for ITS region of phylogenetic tree

jModeltest 0.1 (Posada, 2008) selected TrN+G : Tamura-Nei plus Gamma as the most suitable evolutionary model for ITS region alignment data. Substitution rates were estimated as follows:

freqA = 0.2267

freqC = 0.3031

freqG = 0.2428

freqT = 0.2275

R(a) [AC] = 1.0000

R(b) [AG] = 1.0821

R(c) [AT] = 1.0000

R(d) [CG] = 1.0000

R(e) [CT] = 4.2018

R(f) [GT] = 1.0000

*Note: R(a) [A-C] signifies the rate of change from adenine to cytosine. The nomenclature applies to all other substitution rates

Proportion of invariables sites (sites that cannot change) were estimated to be zero. Variable sites were gamma distributed with α 'shape' parameter = 0.2210, suggesting most sites to be invariable, a considerable amount of sites showing low to moderate substitution rates and a small amount of sites showing high substitution rates. The details of the ITS model selection are shown in Appendix 5.0.

4.3.3 ITS phylogenetic tree

Isolates grouped into 4 major clades. The low divergence and short branching patterns observed in individual major clades suggested the isolates were closely related. Clade A consisted of all the *C. truncatum* that were tentatively identified in the morphology study in Chapter 3. Although isolates B16 and DO6 diverged from the main cluster, their divergence was not significant to support new species.

Clade B consisted of the *C. gloeosporioides* complex with isolates BKM, MO1 and NO1 clustering similar to the type isolates *C. asianum*, and *C. queenslandicum*. The bootstrap value of 0.39 was too low to significantly differentiate these isolates from the two type isolates. Isolates O11 and OO5 formed a significantly separate clade to the other isolates in this complex with a bootstrap value of 0.97. Within this clade isolate 011 clustered with the type isolate of *C. siamense* (0.76), and OO5 clustered with a group of *Colletotrichum* spp type isolates of *C. fruticola*, *C. aeshynomenes* and *C. gloeosporioides* (0.23) however the bootstap values within this subclade were not significant to differentiate these isolates.

Clade C consisted of the *C. acutatum* complex with isolates A13, E15, CO4, A15 and F59 clustering with the type isolate of *C. scovillei*. The strawberry isolates, STP22 and TH5 formed a separate subclade with high bootstrap value of 0.77 to the type isolate of *C. indonensense*; however, there was a weak support (0.27) for differentiation of the chilli and strawberry isolates within this complex.

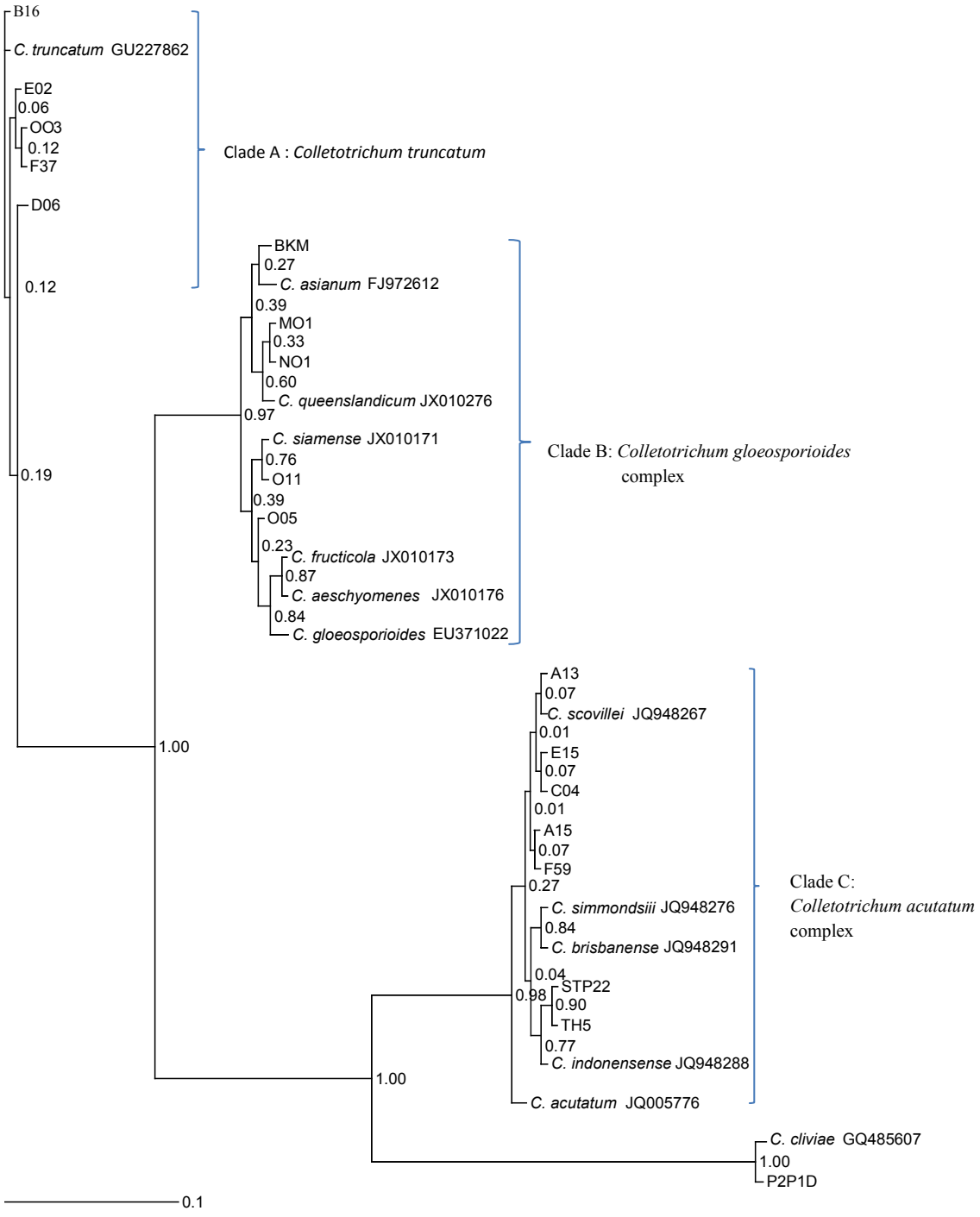


Figure 4.2. ITS 1-5.8S- ITS2 Bayesian trees with numbers on the branches indicate bootstrap and posterior probability value.

4.4 Discussion

Phylogenetic analysis based on ITS gene sequence clearly showed that there were more than three species of *Colletotrichum* pathogenic on chilli in Malaysia. Although the oblong spore type isolates clustered within the *C. acutatum* and *C. gloeosporioides* complexes these were most likely to be species of *C. asianum*, *C. queenslandicum, C. siamense*, *C. scovillei* that caused anthracnose in chilli. This is the first report of these species being identified as pathogens of chilli in Malaysia. Of these only *C. siamense* and *C. scovillei* have been reported as anthracnose pathogens of chilli in Thailand and Indonesia. *Colletotrichum asianum* has been reported as a pathogen of mango and coffee in Thailand; while *C. queenslandicum* has been reported as a pathogen of papaya and persimmon in Australia (Damm *et al*., 2012; Weir *et al*., 2012.).

The *C. truncatum* clade was clearly distinguished with only one species, *C. truncatum* (frequently referred to as *C. capsici*; Damm *et al*, 2009), although there was some divergence with isolates B16 and DO6. Further validation of the identification of these isolates needs to be undertaken by analysing more fungal gene sequences.

The identification of these species needs to be verified using 3 or 4 more fungal gene sequences that can differentiate *Colletotrichum* species of the acutatum and gloeosporioides complexes as described by Cannon *et al*., (2012). According to Cannon *et al*, (2012), the ITS sequence resolves major clades well but possible posterior support was lacking within many major clades especially those in the *C. acutatum* and *C. gloeosporioides* complexes. Not until the identification of these pathogens has been properly validated using multiple gene phylogeny can they be claimed to be the first reports of pathogens in chilli in Malaysia.

Nevertheless, many species of *Colletotrichum* can be differentiated and identified along with spore morphology based on just ITS gene sequence alone (Cai *et al*., 2009; Barimani *et al*., 2013). Isolate BKM clustered close to *C. asianum* and since Prihastuti *et* al. (2009) stated that *C. asianum* could be distinguished by ITS alone then this isolate was most likely *C. asianum*. In contrast, isolates MO1 and NO1 clustered close to *C. queenslandicum* however, the ITS sequences for this species is not readily differentiated from the ITS sequence of *C. fructicola*, *C. siamense*, and *C. tropicale* isolates. *Colletotrichum queenslandicum* has not been reported as a pathogen of chilli thus is is most likely that MO1 and NO1 are in fact *C. siamense* which is a known pathogen of chilli in Thailand. Therefore, it is important that these isolates are identified based on a multi gene phylogeny that includes sequences of TUB2, GAPDH, or GS (Weir *et al*., 2012).

In the *C. acutatum* complex, most of the isolates clustered with the *C. scovillei* clade. The spore morphology of these isolates was similar to the description of the type isolate - cylindrical to clavate with one end round and one end ± acute especially strains that being repeatedly subcultured (Damm *et al*, 2013). *Colletotrichum scovillei* has been reported to cause anthracnose on chilli fruit in Thailand (Than *et al*., 2008; Damm *et al*., 2012).

Isolates from the strawberry host, TH5 and STP22 clustered close with *C. indonensiense* however, because of a weak support (0.27) for differentiation of the chilli and strawberry isolates within this complex, they could in fact be any of several *Colletotrichum* species including *C. simmondsii* which has been reported as a pathogen of strawberry (Damm *et al*., 2012). *Colletotrichum indonensiense* has only been recoded as a pathogen of Eucalyptus spin Indonesia (Damm *et al*., 2012). These isolates need to be identified based on multi gene phylogeny.

Isolate P2P1D from papaya clustered with *C. cliviae*. The isolate had a very wide conidia and was genetically distinct from type isolate species in the *C. gloeosporioides* complex, (Cannon *et al*, 2012). According to Damm *et al*, (2012), *C. cliviae* was a poorly known species that had unusually large conidia. This may be the first report of *C. cliviae* infecting papaya and thus needs to be characterised using multi-gene phylogeny. A final summary of the most likely identity of the 18 isolates after considering both morphology / ITS sequence are presented in Table 4.3.

Table 4.3. Summary of the most likely identity of 18 isolates after considering both morphology / ITS sequence.

Isolate	Complex / Clade	Possible species ID based on morphology / ITS
A13	*C. acutatum*	*C. scovillei*
A15	*C. acutatum*	*C. scovillei*
C04	*C. acutatum*	*C. scovillei*
E15	*C. acutatum*	*C. scovillei*
F59	*C. acutatum*	*C. scovillei*
BKM	*C. gloeosporioides*	*C. asianum*
M01	*C. gloeosporioides*	*C. queenslandicum*
N01	*C. gloeosporioides*	*C. queenslandicum*
O05	*C. gloeosporioides*	*C. queenslandicum*
O11	*C. gloeosporioides*	*C. siamense*
B16	*C. truncatum*	*C. truncatum*
D06	*C. truncatum*	*C. truncatum*
E02	*C. truncatum*	*C. truncatum*
F37	*C. truncatum*	*C. truncatum*
O03	*C. truncatum*	*C. truncatum*
STP22	*C. acutatum*	*Colletotrichum* species
TH5	*C. acutatum*	*Colletotrichum* species
P2P1D	*C. acutatum?*	*C. cliviae*

Chapter 5.0 Identification of pathotypes and cross host pathogenicity of *Colletotrichum* sp.

5.1 Introduction

5.1.1 Pathotypes of *Colletotrichum* on *C. annuum*

There have been several reports of pathotypes of *Colletotrichum* species based on qualitative differences in infection (*C. acutatum* in citrus – You *et al*., 2007; *C. sublineolum* in sorghum – Moore *et al*., 2008; *C. truncatum* in chilli - Montri *et al*. 2009; Mongkolporn *e*t al., 2010) and quantitative differences in severity (*C. lindemuthianum* in bean – Gonzalez-Chavira *et al*., 2004; *C. truncatum* in chilli - Sharma *et al*. 2005; *C. falcatum* in sugarcane – Suman *et al*., 2005) between isolates on specific host genotypes.

According to Taylor and Ford (2007) a pathotype can be defined as a subclass or group of isolates distinguished from others of the same species by its virulence on a specific host (genotype) i.e., a qualitative difference in disease severity. Pathotype also means a physiological response based on the pattern of disease expression across a well-characterized differential set of host lines carrying different resistance genes. A quantitative difference in severity of infection based on lesion size is simply a reflection of the variation of aggressiveness of isolates and does not constitute a true pathotype difference (Taylor & Ford, 2007). Bioassays for pathotyping can provide important biological information when evaluating individual isolates for the range of virulence within a genetically diverse species.

Although *C. annuum* lacks resistance to anthracnose, several wild chilli varieties from *C. baccatum* ‘PBC80’ and ‘PBC81’ and *C. chinense* ‘PBC932’ and ‘CO3865’ have been identified to be highly resistant (immune) to anthracnose (AVRDC, 1998). The resistance derived from PBC932 was differentially expressed at seedling and fruit

stages with three recessive genes *co1*, *co2* and *co3*, identified as being responsible for the resistance at mature green fruit (Pakdeevaraporn *et al*. 2005), ripe fruit and seedling (Mahasuk *et al*. 2009a) respectively.

Pathotypes of each of the major *Colletotrichum* species causing anthracnose in chilli have been identified (Montri *et al*. 2009; Mongkolporn *et al*. 2010). Three pathotypes were identified for *C. truncatum* isolates on differential genotypes of *Capsicum chinense* – PBC932 and C04714. Three pathotypes were also identified for *C. acutatum* on *Capsicum baccatum* - PBC 1422. For *C. gloeosporioides* six pathotypes were identified at the green and five at the red fruit stages on differential genotypes of *C. chinense* – C04714, *C. annuum* – Bangchang, 83-168, Jinda and *C. frutescens* – Kee Noo Suan and Karen (Mongkolporn and Taylor, 2011). However, new taxonomy of the '*acutatum*' and '*gloeosporioides*' complexes have recently recognized that there are different species that cause anthracnose of chilli; hence the differences in host reactions that were attributed to pathotypes in these earlier studies could in fact be host reactions to different species.

It is also important to determine the host range for a particular *Colletotrichum* species as this may have importance in implementing integrated disease control strategies. Host range studies will also assist in understanding the taxonomy of species (Cai *et al*., 2009). Than *et al*. (2008) showed pathotype differences within *C. acutatum* isolates from infected strawberry and chilli fruit. Isolates from chilli were able to infect inoculated fruit of the resistant *C. chinense* genotype 'PBC932', whereas isolates from strawberry were unable to infect this genotype. Both isolates were able to infect the susceptible *C. annuum* chilli genotype 'Bangchang'.

The aim of this Chapter was to identify pathotypes within each of the *Colletotrichum* species isolated from *C. annuum;* and to assess the pathogenicity of isolates of *Colletotrichum* species from papaya and strawberry.

5.2 Materials and methods

5.2.1 Pathotypes of *Colletotrichum* sp.

Isolates were cultured and identified as stated in Chapters 3 and 4. Based on the revised taxonomy from Chapter 4, the isolates from the *C. acutatum* complex were most likely all *C. scovillei* (Cs); whereas the isolates from the *C. gloeosporioides* complex were divided into *C. asianum/ C. queenslandicum* Cag)*;* an unknown *Colletotrichum* sp (C?) and *C. siamense* (Csi). Unfortunately, cultures of isolates A15, E15, F59 (*C. scovillei*) and DO6, EO2 (*C. truncatum*) failed to sporulate thus were not included in these pathogenicity experiments.

Inoculation bioassays were done using spore suspension on several varieties of chilli, *C. annuum* (Kulai, red for mature and green for immature), *C. annuum* (bell pepper, red and yellow for mature and green for immature) and *C. chinense* (yellow for mature and green for immature). Kulai was sourced from Ulu Chuchoh farm in Selangor, bell peppers came from a local market in Kuala Lumpur; while *C. chinense* CO3865 was obtained from AVRDC, Taiwan and plants grown in shade house at University of Malaya.

Spore suspension was made by the aid of a haemocytometer to determine the concentration of spores in the suspension. Conidia were dislodged on the agar plate and 1 ml of the spore suspension pipetted out from the petri dish into a 1.5 ml microcentrifuge tube. An adequate amount of the suspension was then pipetted onto a

haemocytometer and allowed to fill the area under the cover slip via capillary action, ensuring that the mirrored surface was covered for precision counting. At 400X magnification, the counting chamber was divided into 9 large squares, each with a surface area of 1 mm^2 and a depth of 0.1 mm. Each square represented a total volume of 0.1 mm^3. The following was used to obtain the final count of spores in 1 ml of suspension.

Total count by 0.1 mm^3 (chamber depth) / Total surface area counted (each large square 1 mm^2)

Spores per ml of suspension = the average count per square x the dilution factor x 10^4

Both immature green and mature red/yellow stages of chilli fruit were washed with 10% Clorox for 3 minutes, then washed in sterile distilled water several times and dried with sterile paper towels. Wounding took place by using a pin to insert several small holes through the cuticle and epidermis of the fruit. Wounded and non-wounded chilli fruits were then placed in plastic containers, with 100 ml of sterile water to maintain 100% of relative humidity. Inoculations were completed on both wounded and non-wounded fruit by pipetting 6 µl drops with 10^6/ml spore suspension onto the fruit surface – for wounded fruit the inoculum was placed over the wound. Control fruit were inoculated with 10 µl of sterile water. Each test was carried out in the replicates of 5 fruits per isolates.

Containers were covered and kept in darkness at 28°C. Lids of the containers were removed after 48 hour of inoculation, thereafter fruits were incubated for additional 7 days under the same conditions until evaluation (Kim *et al*., 1999).

5.2.2 Pathogenicity of non-host *Colletotrichum* species on *Capsicum*.

Colletotrichum spp isolates from strawberry and papaya were obtained from farms in Cameron Highlands, and market at SS2, Petaling Jaya, Selangor respectively. These isolates were isolated from the fruit, cultured on PDA in petri dishes, incubated for 7 days then evaluated for colony characteristics and conidia morphology. (Please refer chapter 2 and chapter 3 for the materials and methods). Based on ITS gene sequences (Chapter 4) the strawberry isolates (TH5, STP22) were identified as belonging to the *C. gloeosporioides* complex while the papaya isolate (P2P1D) to the *C. cliviae* species.

Both immature green and mature red/yellow stages of chilli fruit were wounded and inoculated with 10^6/ml spore suspension of each isolate as described in section 5.2.1.

5.2.3 Evaluation of anthracnose symptoms

Symptoms were evaluated by observations of lesions that developed on the fruit at the inoculation site at 2, 4, 6 and 8 Day After Inoculation (DAI) and by the description of the anthracnose symptoms at 8 DAI. Disease scores ranged from 0 to 9 and were based on percentage of lesion size to fruit size as described in Table 5.1 according to Montri *et al*., (2009). Pathotypes were identified where qualitative differences in infection (0 vs 1-9 scores) occurred for isolates on a specific chilli genotype. Differences in lesion size were an indication of the levels of aggressiveness of each isolate.

Table 5.1. Anthracnose severity scores on chilli fruits, chilli resistance levels and symptom description.

Score	Resistance level	Symptom details
0	Highly resistant (HR)	no infection
1	Resistant (R)	1-2% of the fruit area shows necrotic lesion or a larger water-soaked lesion surrounding the infection site
3	Moderately resistant (MR)	>2-5% of the fruit area shows necrotic lesion, acervuli may be present, or water-soaked lesion up to 5% of the fruit surface
5	Moderately susceptible (MS)	>5-15% of the fruit area shows necrotic lesion, acervuli present, or water-soaked lesion up to 25% of the fruit surface
7	S, susceptible	>15-25% of the fruit area shows necrotic lesion with acervuli
9	HS, highly susceptible	>25% of the fruit area shows necrosis, lesion often encircling the fruit, abundant acervuli

5.3 Results

None of the non-wounded fruit showed any lesions or symptoms of disease within the time course of the experiments which was in contrast to wounded fruit that showed a range of symptoms with different isolates, thus all results were based on wounded fruit.

5.3.1 Pathogenicity of *Colletotrichum* sp. on wounded mature fruits

Based on the qualitative differential reactions (Table 5.2), in the *Colletotrichum acutatum* clade, only one pathotype of *Colletotrichum scovillei* (PCs) were identified, PCs1. This species only included two isolates A13 and CO4 which were highly virulent on the genotype of *C. annuum* (Kulai), with host reaction ratings of 7. These isolates did not infect the other genotypes tested.

In the C. *gloeosporioides* clade two pathotypes were identified within the *C. asianum* / *C. queenslandicum* species (PCaq1 and PCaq2). This group consisted of BKM (PCaq1) with host reaction rating on Kulai and Bell pepper red of 7, MO1 and NO1 only infecting Kulai with host reactions of 7 and 5 respectively. The unknown *Colletotrichum* sp, OO5, infected only Kulai with host reaction rating of 7; and *C. siamense*, O11 only infected Kulai with a host reaction rating of 5. These latter species could not be assigned pathotype as they were only one isolate of each species.

Three pathotypes were identified within *C. truncatum* as PCt1, PCt2, and PCt3. PCt1 isolate BO4 and OO3, infected *C. annuum* (Kulai) with host reaction ratings of 5 and 7 respectively and (bell pepper yellow or red) with host reaction ratings of 7 and 9 respectively. PCt2 isolates B16 only infected *C. annuum* (Kulai) with host reaction rating of 7. PCt3 isolate F37 infected *C. annuum* (Kulai and bell pepper yellow and

red) with susceptible host reaction rating of 7, 9 and 9 respectively. All isolates of *Colletotrichum* species used in this study showed no infection on the resistant genotypes *C. chinense* (CO3865).

Figure 5.1. Disease diagram representing degrees of infection ranging from scores of 0, 1, 3, 5, 7, and 9 respectively.

Table 5.2. Differential reaction on wounded ripe fruits of *C. annuum* (Kulai), *C. annuum* (bell pepper) and *C. chinense* (CO3865).

Species	Isolates	*Capsicum annuum* (Kulai)		*Capsicum annuum* Bell pepper (Red)		*Capsicum annuum* Bell pepper (yellow)		*Capsicum chinense* (CO3865)		Pathotypes
		Infected area (%)	Host reaction	Infected area (%)	Host reaction	Infected area (%)	Host reaction	Infected area (%)	Host reaction	
C. scovillei	A13	24.9	7	0	0	0	0	0	0	PCs1
C. scovillei	CO4	23.6	7	0	0	0	0	0	0	PCs1
C. asianum or	BKM	23.8	7	21.67	7	0	0	0	0	PCaq1
C. queenslandicum	MO1	21.21	7	0	0	0	0	0	0	PCaq2
C. queenslandicum	NO1	9.6	5	0	0	0	0	0	0	PCaq2
Colletotrichum spp	OO5	21.44	7	0	0	0	0	0	0	PC?
C. siamense	O11	9.6	5	0	0	18.57	7	0	0	PCsi
C. truncatum	BO4	9.1	5	0	0	16.67	7	0	0	PCt1
C. truncatum	B16	23	7	0	0	0	0	0	0	PCt2
C. truncatum	F37	19.12	7	39.6	9	25.37	9	0	0	PCt3
C. truncatum	OO3	17.15	7	29.53	9	0	0	0	0	PCt1

5.3.2 Pathogenicity of *Colletotrichum* sp. on wounded immature fruits

Results of differential reaction on wounded immature fruits are shown in Table 5.3. Two pathotypes of *C. scovillei* weas identified. PCs1 was from isolate A13 which infected only *C. chinense* (CO3865) with a host reaction rating of 9; and PCs2 isolate CO4 was unable to infect any of the genotypes.

In the *C. gloeosporioides* clade two pathotypes were identified within the *C. asianum* / *C. queenslandicum* species (PCaq1 and PCaq2). PCaq1 isolate BKM infected Kulai with a host reaction rating of 7, while the other isolates were unable to infect any of the genotypes.

Three pathotypes were identified within the *C. truncatum* clade, PCt1, PCt2 and PCt3. PCt1 isolates BO4 and OO3 infected *C. annuum* Kulai with host reaction ratings of 7. PCt2 isolate B16 were unable to infect any genotype. PCt3 isolate F37 infected PCt3 isolate F37 infected Kulai and *C. chinense* (CO3865) with a host reaction rating of 7.

Table 5.3. Differential reaction on wounded immature fruits of *C. annuum* (Kulai), *C. annuum* (bell pepper) and *C. chinense* (CO3865).

Species	Isolates	*Capsicum annuum* (Kulai)		*Capsicum annuum* Bell pepper (green)		*Capsicum chinense* (CO3865)		Pathotypes
		Infected area (%)	Host reaction	Infected area (%)	Host reaction	Infected area (%)	Host reaction	
C. scovillei	A13	0	0	0	0	29.17	9	PCs1
C. scovillei	CO4	0	0	0	0	0	0	PCs2
C. asianum or	BKM	16.79	7	0	0	0	0	PCaq1
C. queenslandicum	MO1	0	0	0	0	0	0	PCaq2
C. queenslandicum	NO1	0	0	0	0	0	0	PCaq2
Colletotrichum spp	OO5	0	0	0	0	0	0	PC?
C. siamense	O11	0	0	0	0	0	0	PCsi
C. truncatum	BO4	19.31	7	0	0	0	0	PCt1
C. truncatum	B16	0	0	0	0	0	0	PCt2
C. truncatum	F37	24.19	7	0	0	25.83	7	PCt3
C. truncatum	OO3	16.12	7	0	0	0	0	PCt1

5.3.3 Pathogenicity of non-host *Colletotrichum* species on *Capsicum annuum*

Two isolates from infected strawberries (STP22, TH5) and an isolate from infected papaya (P2P1D) were inoculated onto *Capsicum* spp genotypes to assess pathogenicity. Inoculation test were conducted on mature and immature fruits of *C. annuum* (Kulai and bell pepper) and resistant *C. chinense* cultivar CO3865. None of the isolates were able to infect any of the immature (green) *Capsicum* genotypes. However, on mature fruit isolates TH5 and P2P1D infected only the Kulai genotype fruit (Table 5.4). Isolates from strawberries, TH5 showed infection areas of 19.46% and STP22 showed no infections. Isolates from P2P1D showed infection with 32.29% infected areas. These isolates showed no infection on *C. annuum* (bell pepper red and yellow) and resistant cultivar, *C. chinense* (CO3865).

Table 5.4. Cross inoculation study on wounded ripe fruits of *C. annuum* (Kulai), *C. annuum* (bell pepper) and *C. chinense* (CO3865).

Species	Isolates	*Capsicum annuum* (Kulai)		*Capsicum annuum* Bell pepper (green)		*Capsicum chinense* (CO3865)	
		Infected area (%)	Host reaction	Infected area (%)	Host reaction	Infected area (%)	Host reaction
C. sp	TH5	19.46	7	0	0	0	0
C. sp	STP22	0	0	0	0	0	0
C. cliviae	P2P1D	32.29	9	0	0	0	0

5.4 Discussion

Based on qualitative differences of infection on mature fruit of genotypes of *Capsicum annuum*, one pathotypes were identified for *C. scovillei*, and three for *C. truncatum*. There may also be two pathotypes of the *C. asianum/C. queenslandicum* species however, these species in the *C. gloeosporioides* complex need further gene sequencing to validate the taxonomy before assessing for pathotype differences. On the immature green fruit one pathotypes of *C. scovillei* were identified on the basis that isolate A13 infected the resistant genotype of *C. chinense*, CO3865. Three pathotypes were identified in *C. truncatum* with isolate F37 (pathotype Ct3) infecting CO3865. Again there may be two pathotypes of the *C. asianum/ C. queenslandicum* species with only isolate BKM infecting genotype Kulai.

Pathotypes of *Colletotrichum* species causing anthracnose in chilli have been previously identified in *C. truncatum* isolates on differential genotypes of *Capsicum chinense* – PBC932 and C04714 (Montri *et al*. 2009; Mongkolporn *et al*. 2010). Pathotypes were also identified in *C. acutatum* on *Capsicum baccatum* - PBC 1422 and in *C. gloeosporioides* on differential genotypes of *C. chinense* – C04714, *C. annuum* – Bangchang, 83-168, Jinda and *C. frutescens* – Kee Noo Suan and Karen (Mongkolporn and Taylor, 2011). However, new taxonomy of the '*acutatum*' and '*gloeosporioides*' complexes casts doubt on the nature of the pathotypes in these earlier studies which could in fact be host reactions to different species.

Infection of the resistant *C. chinense* genotype CO3865 by an isolate of *C. scovillei* and *C. truncatum* indicated that resistance in this genotype was stronger in mature green fruit compared to the immature fruit. These results also indicate that two pathotypes may have evolved avirulence genes that were not recognised by the host thus allowing infection, or the isolates of the pathogen had developed pathogenicity genes

that overcame the defence barriers of the host. The change in resistance of *C. chinense* genotypes has been reported in previous studies by Pakdeevaraporn *et al*. (2005), Mahasuk *et al*. (2009a) and Mongkolporn *et al*. (2012). The resistance in PBC932 was shown to be differentially expressed at different physiological ripening stages of the fruit and involved three recessive genes.

Isolate A13 was the most virulent *C. scovillei* isolate as it infected mature fruit of *C. annuum* and immature fruit of *C. chinense*. However, this isolate was not able to infect immature fruit of *C. annuum* which is strange as it is unlikely that these immature fruit of the susceptible genotype Kulai have any resistant genes. Further studies into infection in these fruits are required. In the *C. truncatum* isolates F37 was the most virulent as it was able to infect the mature and immature fruit of all *C. annuum* genotypes and the immature fruit of the *C. chinense* genotype. There was one exception with this isolate unable to infect immature fruit of *C. annuum* bell pepper green. This work needs repeating to validate this result.

The strawberry and papaya isolates (TH5 and P2P1D) respectively of *Colletotrichum* were only able to infect the mature susceptible chilli genotype Kulai after wounding. In contrast, no infection occurred on non-wounded and mature chilli fruit. This demonstrated the poor cross host pathogenicity of these *Colletotrichum* isolates, and the importance of the cuticle to prevent infection. In contrast, Than *et al*. (2008) showed that a susceptible *Capsicum*, spp genotype (Bangchang) could be infected by strawberry isolates of *Colletotrichum acutatum* after both wounding and non-wounding of the cuticle. However the strawberry *C. acutatum* isolates could not infect the resistant chilli genotype (PBC932) although the chilli isolates of *C. acutatum* could but only after wounding.

It is important to note that the Malaysian isolates of *C. acutatum* isolated from strawberry were most likely different species to the strawberry isolates in Than *et al*. (2008). In fact the isolates from strawberry and chilli used in the Than *et al* (2008) study were most likely different *Colletotrichum* species; making the interpretation and comparison of the results confusing.

It was not surprising that only wounded mature chilli fruit were infected. The cuticle has been shown to play an important role in preventing infection of chilli by *Colletotrichum truncatum* (Taylor *et al*. 2011). There are many resistance genes in *Caspicum spp.* that are expressed at different physiological ripening stages of the fruit (Mongkolporn *et al*., 2010).

Only one of the two strawberry isolates of *Colletotrichum* spp were able to infect *Capsicum* genotype Kulai which may indicate that either these are different pathotypes or since the taxonomy of these isolates were not resolved they could have been different species. Strawberries are known to be infected by several *Colletotrichum* species (Damm *et al*., 2012). *C. cliviae* is a poorly known species (Damn ***e****t al*., 2012), with unusually large conidia thus more work is needed to validate the identification and understanding of the morphology of this species, This may be the first report of *C. cliviae*, found on papaya in Malaysia.

Chapter 6.0 General discussion

6.1 Morphological and molecular identification

At the beginning, this research was basically represented by three main species of *Colletotrichum*, which are the *C. acutatum*, *C. gloeosporioides* and *C. truncatum*. In this study, the most commonly isolated fungal species from infected chilli fruits was *Colletotrichum truncatum*. Lin *et al*. (2002) and Voorrips *et al*. (2004) stated that the causal pathogens in Thailand and Indonesia were primarily *C. gloeosporioides* and *C. truncatum.* Most of the species found in Peninsular Malaysia are *Colletotrichum truncatum* and *C. acutatum*. In addition, the high diversity of *C. truncatum* found in Peninsular Malaysia showed that this species is very well adapted to this environment.

Colletotrichum acutatum and *C. gloeosporioides* are two members of the genus of *Colletotrichum* that are most commonly associated with fruit rots. *Colletotrichum gloeosporioides* is considered a cumulative species and is found on a wide variety of fruits, including almond, apple, avocado, citrus, mango olive, and strawberry (Fitzell, 1979; Sutton, 1992; Freeman & Shabi, 1996; Freeman *et al*., 1998; Martín & García-Figueres, 1999; Arauz, 2000; Timmer & Brown, 2000). *Colletotrichum acutatum* clade and *C. gloeosporioides* clades are morphologically very similar and due to because of their overlapping host range and the extensive variability, these isolates showed in the culture, had been very difficult to separate them based on traditional taxonomic methods. Buddie *et al*, 1999 stated that secondary conidia formed from germ tube are irregular and smaller, with frequent sub-cultured may have more variable appearance than recent stock. In addition, (Nirenberg *et al*, 2002) observed that shape of the conidia is differed from that formed in acervuli and aerial mycelium. Due to this variation they are often being incorrectly place into synonym with other *Colletotrichum* species primarily *C. gloeosporioides.*

Generally, the morphology raises problems in the identification, and has long been difficult to identify due to limited morphological characters. Each species of *Colletotrichum* in this study, were found to have many morphological patterns or cultural characteristics that agree with Hyde *et al*. (2009a). Isolates of *Colletotrichum acutatum* have 4 different colony characteristics within the same species and host. According to (Johnston & Jones, 1997; Prihastuti *et al*, 2009) cultural morphology are useful for grouping isolates when samples locally, but break down within a clade when sampled globally. Storage will caused culture to become stale and losing the ability to produce well-differentiated acervuli, conidia and perithecia. In our study, some of the isolates such as DO6, F59 and E15 have very little spores due to poor sporulation that might be caused by storage. This variation makes morphological characteristics difficult to interpret for accurate identification (Damm *et al*., 2012).

6.2 Limitations of morphology and molecular characteristics

In the present, a number of limitations need to be improved to produce a more straightforward and quick identification of the species in disease control and plant breeding. Since morphological characteristics give many problems and inaccuracies, molecular evidences are used as alternative methods.

Adaskaveg & Hartin (1997) and Hyde *et al*. (2009) stated that phenotypic plasticity maybe due to the lack of standardization of cultural technique that leads to taxonomic confusion. There is a need in standardising the incubation parameters, inoculation methods, replicates of the isolates, conidial counts and measurements, colony characteristic recorded and appressoria culture technique (Cai *et al*, 2009).

Molecular evidence using ITS region is proven to be significance for inter and intra specific phylogenetic studies. The isolates of this study were analyse with ITS

sequences to confirm the *Colletotrichum* species aggregate (Cannon *et al*., 2008). The shape and size of the conidia could be used for evaluation but depended on the growth condition (Cai, *et al*., 2009). Morphological characters in identifying fungi are easy to observe and recorded (Talbot, 1971) but they may not reflect the phylogenetic relationships as they are often subject to plasticity, parallelism and reversal (homoplasy) (Judd *et al*., 2002). For example, Prihastuti *et al*. (2009) highlighted the problems associated with inadequate knowledge and incorrect or inaccurate naming of the species using three selected species, *C. dematium*, *C. destructivum* and *C. fragariae*. Morphological data gave very confusing identification of the species studied. In this study, most of the sequences blasted showed inaccuracy due to the information in the genbank might be wrong or mismatch. Example, isolate P2P1D were blasted using GenBank, and revealed to be *C. gloeosporioides*, *C. cliviae*, *Colletotrichum* sp, and *C. magna*. Due, to its unusually large conidia, we suspected that this might be another species under *C. gloeosporioides* clade. This species were aligned with type species to identify them and it matches *C. cliviae.*

The efforts for identification of *Colletotrichum* have generated many methods in facilitating the efficiency and accuracy including the use of a set of genes such as ITS, 18S, LSU, SSU and etc. Molecular characters and tools are undoubtedly an indispensible part of fungal systematic (Shenoy, *et al*., 2007). According to Huang *et al*. (2009), genetic analysis of ITS1-5.8S-ITS2 sequences was a good agreement between morphological and ITS sequence-based approaches. Identification has long been difficult due to limited morphological characters. Single gene identification has not been successful to delineate the species due to erroneous data in Genbank. Many morphological patterns or characteristics found in a few isolates in this study also reported by Hyde *et al*. (2009b). Phylogenetic tree construction in this study was

estimated with a model of evolution and the tree is very much dependent on the sequence alignment. An inaccurate tree might be obtained if the sequence is not properly carried out.

Further study on more aspect of identification such as DNA barcoding could provide a more established and accurate universally accessible identification system. This system could solve a major problem like *Colletotrichum* species where they are widely distributed around the world. This will contribute very much to the disease control and management.

6.3 Pathotypes characters of *Colletotrichum*

In this study, isolates were injected with spore suspension but not all chilli were emerged with lesion. This might be due to loss of psathoginicity of the isolates after subsequent culturing. This may be partly due to the wide host range of a number of *Colletotrichum* species and the fact that several *Colletotrichum* species may be associated with a single host (Freeman *et al*., 1998).

According to Sutton (1980); Dodd *et al*. (1992); Cannon *et al*. (2008), stated that *Colletotrichum gloeosporioides* Penz is the most predominant *Colletotrichum* pathogen that attacked about 470 different host genera. (Lin *et al*. (2002) & Voorrips *et al*. (2004), stated that primarily causal pathogens in Thailand and Indonesia are *C. gloeosporioides* and *C. truncatum*. AVRDC (2002) has identified five accession of peppers (*Capsicum chinense* CO4554, PBC 932, *Capsicum baccatum* PBC 80, PBC 81, PBC 133) that are resistant *C. gloeosporioides* attack on both green and red stages, meanwhile *C. truncatum* mainly attack red stage (Park *et al*., 1990b). In this study, main findings that *C. scovillei* is not that aggressive compared to *C. gloeosporioides clade* and *C. truncatum*. Disease in farms through the data farm survey found that no much of

C. gloeosporioides clades were found. Most of the species found in Peninsular Malaysia are *Colletotrichum truncatum* and *C. acutatum* clade species. According to Yun *et al* (2009), stated that, most dominant pathogen found to be from *C. gloeosporioides* clade as most sample were obtained in Sabah. In this study, all isolates were obtained from farms and not from the market thus less infection from external pathogen.

Different hosts and maturity of the host are important to access the expression of resistance to *Colletotrichum* species. The wounded or drop method showed to be very useful to select resistant varieties of *Capsicum annuum* from susceptible varieties (Lin *et al*., 2002). According to Damm *et al*. (2012), species that found to be pathogenic on one host and can be isolated from other host following opportunistic colonisation of senescing tissue. Multiple *Colletotrichum* spp. asssociated with single host likely to have a variety of lifestyles, pathogens on healthy tissue that invades and cause minor disease when host is under stress, latent infections following to senescence of the host tissue or ripening of host fruits due to senescing or endophyte species that sporulate on death host tissue. This characteristic were able to caused several *Colletotrichum* spp to established either on a single host or a range of host and this become a large part of the confusion surrounding species limits within the *Colletotrichum.*

In this study, some of the *C. scovillei* did not emerge with lesion due to the quiescent of the isolates. Some species were pathogenic on one host and can be isolated from others following the opportunistic colonisation on senescing tissue. The occurrence and relevance of each stage in the infection process may vary depending on the conditions of growth, the host tissue, the particular species, or the fungal isolate (Bailey *et al*., 1992; Zulfiqar *et al*., 1996; Diéguez-Uribeondo *et al*., 2003). Likewise, *C. acutatum* has also been reported to infect a large number of fruit crops (Freeman *et* al., 1998; Martín & García-Figueres, 1999; Adaskaveg & Förster, 2000; Yoshida &

Tsukiboshi, 2002). *C. acutatum* and *C. gloeosporioides* are morphologically very similar and because of their overlapping host ranges and the extensive variability that their isolates show in culture, it has been very difficult to separate them by traditional taxonomical methods. Cross inoculation test showed that *Colletotrichum* has wide range of host which could infect *Capsicum annuum* which is a susceptible variety. In this study, only two isolates of strawberry collected from the farms are used as representative for further analysis. These two isolates showed slight infection with disease score of 7 and 0 on isolate TH5 and STP22. However according to Xiao *et al* (2004), pathogenicity test in artificial conditions were not conclusive support compared to under natural condition. Based on field observation and laboratory data on susceptibility to anthracnose, cultivars were classified into three categories; highly susceptible, moderately susceptible, and resistant. Due to cultivar reactions varied continuously from highly susceptible to highly resistant, significant differences were found in susceptibility among cultivars within each category. The use of less susceptible or resistant cultivars is the best way to control many plant diseases. Fresh wounds were more susceptible, with susceptibility decreasing as wounds aged.

6.4 Limitation in the present study on pathotypes work and future improvement

In this study, most of the results are not that accurate as this in-vitro incubation was not similar to the actual environment. Due to the problems of optimizating the temperature and humidity in the lab as in the field, these results might not be that accurate and lead to no infection due to the temperature that might be a bit lower and humidity that is not high enough. Pathogenicity test should be done in a proper inoculation room with temperature and humidity was properly controlled with less fluctuation.

With regards to chilli farming in Malaysia, more cultivar of resistant should be introduced for commercial use and consumption. This is because the damage is lesser and disease control is moderately done to reduce the usage of fungicide to prevent infections and disease on susceptible cultivar. This could help the management of farms to be more environmentally friendly by reducing the use of chemical.

Chapter 7 Conclusion

Morphological identification have identified tentatively 3 species of *Colletotrichum* as *C. acutatum*, *C. gloeosporioides* and *C. truncatum* based on growth rate and conidia shape and size. *Colletotrichum gloeosporioides* had the fastest growth compared to *C. acutatum* and *C. truncatum*. *C. acutatum* has the slowest growth rate. Growth rate and conidia size can be the criteria for identification and differentiation, but morphology was not a good key for identification as mentioned in (Than *et* al., 2008). Thus, molecular methods were applied for more accurate identification of the samples studied.

However, subsequent molecular identification has differentiated the complex *C. acutatum* and *C. gloeosporiodes* to several species. We have confirmed the identities of the *Colletotrichum* isolates using ITS region. *C. truncatum* was identified as predicted by its morphology. Our isolates that were identified as *C. gloeosporioides* complex consist of tentatively *C. asianum*, *C. siamense*, and *C. queenslandicum*, should be further verified using multigene phylogeny. *C. acutatum* complex consist of *C. scovillei*. The molecular identification has aided the identification and classification. *C. cliviae* was also identified on papaya as they are the first report in Malaysia.

Pathotypes work in this study identified only one pathotypes, PCs1 found on *C. scovillei* and three pathotypes for *C. truncatum,* (PCt1, PCt2 and PCt3). *C gloeosporioides* complex identified 4 pathotypes which consist of (PCaq1, PCaq2, PC? And PCsi) but these need further gene sequencing more ccurate verification. More work need to done for validation and identification as they are not clearly differentiated by only ITS.

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Appendix 1.0 General solution, culture media, DNA extraction solutions, oligonucleotide primers, PCR reagents, agarose gels, solutions for electrophoresis and commercial kits used in this study.

A. General solutions

Generally reagents and chemicals were purchased from Sigma Aldrich® , USA; Fluka, Germany; Finnzymes, Finland; Agarose gels and electrophoresis solution from Promega, USA.

Ethanol, 70% (v/v)

Denatured ethanol 70ml

Purified distilled water was added to a final volume of 100mL

B. Culture media

Difco ™ Potato Dextrose Agar (PDA) plates (Becton, Dickson and company, France)

Difco ™ Potato Dextrose Agar 39g

Potato starch	4.0g
Dextrose	20.0g
Agar	15.0g

Sterilized distilled water was added to final volume of 1L. Media was heated to dissolve the powder. The micture were autoclaved at 121ºC, 27 p.s.i for 20 minutes. Media was allowed to cool to 50ºC before being poured into the 85mm petri dishes. Once the media hardened, they were stored in cool and dry place.

C. Oligonucleotide primers

Primers used for PCR amplification of the nuclear ITS region were ITS5 – ITS4 for the ITS1-5.8S-ITS2 region.

Table A 1.1 Primers for PCR of ITS

Primer	Sequence	Reference
ITS5 (forward primer)	5'GGAAGTAAAAGTCGTAACAAGG-3'	White *et al*., 1990
ITS4 (reverse primer)	5'-TCCTCCGCTTATTGATAGC – 3'	White *et al*., 1990

D. PCR reagents

DyNAzyme™ II DNA Polymerase Reaction buffer (10X) (Finnzymes, Finland)

10 mM Tris-HCI, pH 8.8 at 25ºC

1.5 mM $MgCl_2$,

50 mM KCl

0.1% Triton X-100

E. Agarose gel and solution for electrophoresis

Ethidium Bromide

Tris-Borate-EDTA (TBE) agarose gel, 1.3%

1x TBE was added to a final volume of 100mL and the mixture was heated for 2 minutes until the agarose powder dissolved completely.

Tris-Borate-EDTA (TBE) buffer, 10x concentration

F. Commercial kits

Qiagen DNeasy extraction Kit

Procedure

1. For disruption using the TissueRuptor, follow step 2; for disruption using the TissueLyser, follow steps 3–6.

Alternatively, plant or fungal tissue can be ground to a fine powder under liquid nitrogen using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Proceed immediately to step 7.

2. TissueRuptor procedure: Place the sample material (≤100 mg wet weight or ≤20 mg lyophilized tissue) into a 2 ml microcentrifuge tube. Add liquid nitrogen to the tube, and freeze the sample for 30 s. Keep the sample submerged in liquid nitrogen, and disrupt for approximately 30 s at full speed. Allow the liquid nitrogen to evaporate, and proceed immediately to step 7.

Alternatively, fresh or lyophilized material can be directly disrupted in lysis buffer (after step 7) without using liquid nitrogen, but this may cause shearing of high molecular- weight DNA. We do not recommend disrupting frozen material in lysis buffer as this can result in low yields and degraded DNA.

3. TissueLyser procedure: Place the sample material (_100 mg wet weight or _20 mg lyophilized tissue) into a 2 ml safe-lock microcentrifuge tube, together with a 3 mm tungsten carbide bead. Freeze the tubes in liquid nitrogen for 30 s.

When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

4. Place the tubes into the TissueLyser Adapter Set 2 x 24, and fix into the clamps of the TissueLyser. Immediately grind the samples for 1 min at 30 Hz.

5. Disassemble the adaptor set, remove the tubes, and refreeze in liquid nitrogen for 30 s.

When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

6. Repeat step 4, reversing the position of the tubes within the adaptor set. Proceed immediately to step 7.

To prevent variation in sample homogenization, the adaptor sets should be

removed from the TissueLyser and disassembled after the first disruption step. For the second disruption step, the adaptor sets should be reassembled so that the tube order is reversed. Rotating the racks of tubes in this way ensures that all samples are thoroughly and equally disrupted.

Note: The majority of plant tissue is ground to a fine powder after 2 disruption

steps, however, for some materials one disruption step may be sufficient. Other tissues, such as seeds and roots, may require disruption steps. Optimization of the disruption procedure may be required for some plant material.

7. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant or fungal tissue and vortex vigorously.

No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA. In rare cases, where clumps cannot be removed by pipetting and vortexing, a disposable micropestle may be used.

Note: Do not mix Buffer AP1 and RNase A before use.

8. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation inverting tube.

This step lyses the cells.

9. Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.

This step precipitates detergent, proteins, and polysaccharides.

10. Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).

Some plant materials can generate very viscous lysates and large amounts of

precipitates during this step. This can result in shearing of the DNA in the next step (see "Lysate filtration with QIAshredder", page 21). In this case, optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at 20,000 x g (14,000 rpm). After centrifugation, apply supernatant to QIAshredder Mini spin column and continue with step 11.

11. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm).

It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder Mini spin column. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 12.

12. Transfer the flow-through fraction from step 11 into a new tube (not supplied)

without disturbing the cell-debris pellet.

Typically 450 µl of lysate is recovered. For some plant species less lysate is recovered. In this case, determine the volume for the next step.

13. Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting.

For example, to 450 µl lysate, add 675 µl Buffer AP3/E. Reduce the amount of

Buffer AP3/E accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AP3/E, but this will not affect the DNeasy procedure.

Note: Ensure that ethanol has been added to Buffer AP3/E. See "Things to do

before starting", page 24.

Note: It is important to pipet Buffer AP3/E directly onto the cleared lysate and to mix immediately.

14. Pipet 650 µl of the mixture from step 13, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at _6000 x g (corresponds to _8000 rpm for most microcentrifuges), and discard the flow-through.* Reuse the collection tube in step 15.

15. Repeat step 14 with remaining sample. Discard flow-through* and collection tube.

Appendix 2.0 Descriptive of growth rate of *Colletotrichum* species

Breakdown table of Descriptive statistics

Species	Isolates	n	Mean growth rate (mm/day) Mean ± SD
C. acutatum	A13	6	10.12±2.07
C. acutatum	A15	6	5.55±0.50
C. acutatum	CO4	6	5.95±0.20
C. acutatum	E15	6	5.78±0.20
C. acutatum	F59	6	5.74±0.06
C. truncatum	BO4	6	9.02±0.31
C. truncatum	B16	6	6.85±0.42
C. truncatum	DO6	6	8.24±0.44
C. truncatum	EO2	6	6.29±0.13
C. truncatum	F37	6	7.29±0.24
C. truncatum	OO3	6	8.33±0.44
C. gloeosporioides	BKM	6	11.19±0.20
C. gloeosporioides	MO1	6	11.43±0.00
C. gloeosporioides	NO1	6	11.43±0.00
C. gloeosporioides	OO5	6	10.12±2.07
C. gloeosporioides	O11	6	11.43±0.00

LSD test on Mean growth rate

Species	Mean growth rate (mm/day)		
	6.62	7.67	11.12
C. acutatum		0.003152	0.000000
C. truncatum	0.003152		0.000000
C. gloeosporioides	0.000000	0.000000	

Appendix 3.0 Descriptive statistic of conidia length and width

Isolates	Length means (µm) (N=40)	Width means µm) (N=40)	Ratio means (µm) (N=40)	Tentative species identification
A13	9.27±1.03	2.93±0.27	3.18±0.42	*C.acutatum*
A15	9.33±1.05	3.46±0.31	2.71±0.32	*C.acutatum*
C04	10.63±0.95	2.58±0.32	4.18±0.66	*C.acutatum*
E15	9.41±1.19	3.48±0.36	2.72±0.35	*C.acutatum*
F59	9.98±1.03	2.86±0.21	3.52±0.47	*C.acutatum*
BKM	11.99±1.30	4.66±1.11	2.72±0.67	*C. gloeosporioides*
N01	14.19±0.78	3.67±0.59	4.16±2.16	*C. gloeosporioides*
O05	13.99±1.06	3.89±0.29	3.63±0.50	*C. gloeosporioides*
O11	14.64±0.83	3.51±0.34	4.20±0.47	*C. gloeosporioides*
M01	14.10±0.86	3.66±0.22	3.87±0.33	*C. gloeosporioides*
B04	21.36±1.45	3.11±0.27	6.93±0.77	*C. truncatum*
B16	24.48±1.66	2.89±0.23	8.55±1.04	*C. truncatum*
D06	24.41±1.23	2.93±0.14	8.32±1.02	*C. truncatum*
E02	23.72±1.94	3.02±0.29	7.91±0.94	*C. truncatum*
F37	20.61±2.02	3.93±1.96	6.16±2.02	*C. truncatum*
O03	21.82±1.94	2.76±0.28	7.99±1.16	*C. truncatum*

Isolates															
Mean length (µm)	9.2660	9.3250	10.630	9.4052	9.9970	11.999	14.190	13.998	14.637	14.104	21.359	24.477	23.716	20.615	21.821
A13		0.843726	0.000006	0.641770	0.014840	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
A15	0.843726		0.000015	0.788602	0.025059	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
CO4	0.000006	0.000015		0.000048	0.034774	0.000006	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
E15	0.641770	0.788602	0.000048		0.048394	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
F59	0.014840	0.025059	0.034774	0.048394		0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
BKM	0.000000	0.000000	0.000006	0.000000	0.000000		0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
NO1	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000		0.520716	0.135669	0.773215	0.000000	0.000000	0.000000	0.000000	0.000000
OO5	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.520716		0.033028	0.723224	0.000000	0.000000	0.000000	0.000000	0.000000
O11	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.135669	0.033028		0.075189	0.000000	0.000000	0.000000	0.000000	0.000000
MO1	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.773215	0.723224	0.075189		0.000000	0.000000	0.000000	0.000000	0.000000
BO4	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000		0.000000	0.000000	0.013162	0.122447
B16	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000		0.011275	0.000000	0.000000
EO2	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.011275		0.000000	0.000000
F37	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.013162	0.000000	0.000000		0.000062
OO3	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.122447	0.000000	0.000000	0.000062	

Isolates															
Mean width (µm)	2.9295	3.4595	2.5825	3.4782	2.8575	4.6630	3.6740	3.8875	3.5135	3.6600	3.1060	2.8857	3.0210	3.9257	2.7600
A13		0.000310	0.017839	0.000189	0.622246	0.000000	0.000000	0.000000	0.000072	0.000001	0.227394	0.764646	0.531274	0.000000	0.246341
A15	0.000310		0.000000	0.897902	0.000043	0.000000	0.142499	0.003520	0.711741	0.170378	0.015817	0.000096	0.002796	0.001488	0.000002
CO4	0.017839	0.000000		0.000000	0.060233	0.000000	0.000000	0.000000	0.000000	0.000000	0.000367	0.038318	0.002796	0.000000	0.224775
E15	0.000189	0.897902	0.000000		0.000025	0.000000	0.180714	0.005249	0.809383	0.213883	0.011072	0.000057	0.001832	0.002287	0.000001
F59	0.622246	0.000043	0.060233	0.000025		0.000000	0.000000	0.000000	0.000009	0.000000	0.089418	0.846707	0.263443	0.000000	0.504708
BKM	0.000000	0.000000	0.000000	0.000000	0.000000		0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000001	0.000000
NO1	0.000000	0.142499	0.000000	0.180714	0.000000	0.000000		0.144366	0.272295	0.923674	0.000112	0.000000	0.000009	0.085317	0.000000
OO5	0.000000	0.003520	0.000000	0.005249	0.000000	0.000000	0.144366		0.010702	0.119886	0.000000	0.000000	0.000000	0.793514	0.000000
O11	0.000072	0.711741	0.000000	0.809383	0.000009	0.000000	0.272295	0.010702		0.316285	0.005445	0.000020	0.000796	0.004929	0.000000
MO1	0.000001	0.170378	0.000000	0.213883	0.000000	0.000000	0.923674	0.119886	0.316285		0.000164	0.000000	0.000014	0.069362	0.000000
BO4	0.227394	0.015817	0.000367	0.011072	0.089418	0.000000	0.000112	0.000000	0.005445	0.000164		0.132122	0.560836	0.000000	0.018169
B16	0.764646	0.000096	0.038318	0.000057	0.846707	0.000000	0.000000	0.000000	0.000020	0.000000	0.132122		0.354849	0.000000	0.389635
EO2	0.531274	0.002796	0.002796	0.001832	0.263443	0.000000	0.000009	0.000000	0.000796	0.000014	0.560836	0.354849		0.000000	0.074475
F37	0.000000	0.001488	0.000000	0.002287	0.000000	0.000001	0.085317	0.793514	0.004929	0.069362	0.000000	0.000000	0.000000		0.000000
OO3	0.246341	0.000002	0.224775	0.000001	0.504708	0.000000	0.000000	0.000000	0.000000	0.000000	0.018169	0.389635	0.074475	0.000000	

Appendix 4.0 Alignment of the ITS1-5.8S-ITS2 region of 31 taxa of *Colletotrichum*. Total length was 496 sites.

```
                          1111111111222222222233333333344444444445
Taxon node        12345678901234567890123456789012345678901234567890
------------------------------------------------------------------------
B16               -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
E02               -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
GU227862          -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
O03               -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
F37               -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
D06               -CATTACTGAGTTACCGCTC--ATCAACCCTTTGTGAACATACCT-TAAC
BKM               -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
FJ972612          -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
MO1               -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
NO1               -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
JX010276          -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
JX010171          -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
O11               -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
O05               -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTATAAC
JX010173          -CATTACTGAGTTTACGCTC--TATAACCCTTTGTGAACATACCTATAAC
JX010176          -CATTACTGAGTTTACGCTC--TATAACCCTTTGTGAACATACCTATAAC
EU371022          -CATTACTGAGTTTACGCTC--TACAACCCTTTGTGAACATACCTACAAC
A13               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
A15               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
E15               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
F59               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
C04               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
JQ948267          -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
JQ948276          -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
JQ948291          -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
STP22             -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
TH5               -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
JQ948288          -CATTACTGAGTTACCGCTC--TATAACCCTTTGTGAACATACCT--AAC
JQ005776          -CATTACTGAGTTACCGCTC--TACAACCCTTTGTGAACATACCT--AAC
GQ485607          -------------------------ACCCTTTGTGA-CATACCC-CAAA
P2P1D             -CATTATCGAGTTACCGCTCCTTATAACCCTTTGTGAACATACCC-CAAA
```

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                  5555555556666666666777777777788888888889999999990
                  1234567890123456789012345678901234567890123456789
-------------------------------------------------------------------
B16               TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
E02               TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
GU227862          TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
O03               TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
F37               TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
D06               TGTTGCTTCGGCGGGTAGGCGTCCCCT-AAAAAGGACG--TCTCCCGGCC
BKM               TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
FJ972612          TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---ACTCCCGGCC
MO1               TGTTGCTTCGGCGGGTAGG-GTCTCC------GTGAC---CCTCCCGGCC
NO1               TGTTGCTTCGGCGGGTAGG-GTCTCC------GTGAC---CCTCCCGGCC
JX010276          TGTTGCTTCGGCGGGTAGG-GTCTCC------GTGAC---CCTCCCGGCC
JX010171          TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
O11               TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
O05               TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
JX010173          TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
JX010176          TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
EU371022          TGTTGCTTCGGCGGGTAGG-GTCTCC------GCGAC---CCTCCCGGCC
A13               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
A15               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
E15               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
F59               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
C04               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
JQ948267          CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
JQ948276          CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCTTCCCCTCCCGGC
JQ948291          CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
STP22             CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
TH5               CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
JQ948288          CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
JQ005776          CGTTGCTTCGGCGGGCAGGGGAAGCCTCTCGCGGGCCT-CCCCTCCCGGC
GQ485607          CGTTGCCTCGGCGGGCAGCCGGAGCCC-----AGCTCCGTCGCCCGGAGC
P2P1D             CGTTGCCTCGGCGGGCAGCCGGAGCCC-----AGCTCCGTCGCCCGGAGC
```

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                 1111111111111111111111111111111111111111111111111
                 0000000001111111111222222222233333333334444444445
                 1234567890123456789012345678901234567890123456789
-------------------------------------------------------------------
B16              CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
E02              CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
GU227862         CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
O03              CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
F37              CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
D06              CTCTCCCGTC----CGCGGGTGGGGCGCCCGCCGGAGGATAACCAAACTC
BKM              TCCCGCCCCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
FJ972612         TCCCGCCCCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
MO1              TCCCGCCCCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
NO1              TCCCGCCCCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
JX010276         TCCCGCCCCCC--GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
JX010171         TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
O11              TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
O05              TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
JX010173         TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
JX010176         TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
EU371022         TCCCGCCTCC---GGGCGGGTCGG-CGCCCGCCGGAGGATAACCAAACTC
A13              GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
A15              GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
E15              GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
F59              GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
C04              GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
JQ948267         GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
JQ948276         GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
JQ948291         GCCGGCCCC-ACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
STP22            GCCGGCCCCCACCAGGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
TH5              GCCGGCCCCCACCAGGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
JQ948288         GCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
JQ005776         GCCGGCCCC-ACCACGGGGACGGGGCGCCCGCCGGAGGA-AACCAAACTC
GQ485607         CGCCGTCTC-------GGCGCGCCCCACCCGCCGGCGGACCACCAAACTC
P2P1D            CGCCGTCTC-------GGCGCGCCCCACCCGCCGGCGGACCACCAAACTC
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B16               TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
E02               TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
GU227862          TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
O03               TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
F37               TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
D06               TGATTTAACGACGTTTCTTCTGAGTGACACAAGCAAATAATCAAAACTTT
BKM               TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
FJ972612          TGATGTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
MO1               TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
NO1               TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
JX010276          TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
JX010171          TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
O11               TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
O05               TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
JX010173          TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
JX010176          TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
EU371022          TGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
A13               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
A15               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
E15               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
F59               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
C04               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
JQ948267          TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
JQ948276          TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
JQ948291          TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
STP22             TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
TH5               TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
JQ948288          TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
JQ005776          TATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTT
GQ485607          TATTTAAACGACGTCTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTT
P2P1D             TATTTAAACGACGTCTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTT
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B16              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
E02              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GU227862         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
O03              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
F37              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
D06              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
BKM              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
FJ972612         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
MO1              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
NO1              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JX010276         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JX010171         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
O11              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
O05              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JX010173         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JX010176         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
EU371022         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
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A15              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
E15              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
F59              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
C04              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JQ948267         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JQ948276         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JQ948291         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
STP22            TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
TH5              TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JQ948288         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
JQ005776         TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GQ485607         TAACAATGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
P2P1D            TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
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B16               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
E02               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
GU227862          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
O03               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
F37               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
D06               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
BKM               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
FJ972612          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
MO1               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
NO1               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JX010276          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JX010171          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
O11               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
O05               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JX010173          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JX010176          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
EU371022          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
A13               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
A15               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
E15               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
F59               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
C04               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JQ948267          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JQ948276          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JQ948291          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
STP22             GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
TH5               GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JQ948288          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
JQ005776          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
GQ485607          GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
P2P1D             GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
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E02              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
GU227862         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
O03              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
F37              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
D06              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
BKM              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTTTTCGAGCGTCATT
FJ972612         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
MO1              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
NO1              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
JX010276         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
JX010171         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
O11              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
O05              CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
JX010173         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
JX010176         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
EU371022         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
A13              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
A15              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
E15              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
F59              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
C04              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
JQ948267         CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
JQ948276         CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
JQ948291         CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
STP22            CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
TH5              CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
JQ948288         CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
JQ005776         CACATTGCGCTCGCCAGCATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
GQ485607         CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
P2P1D            CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
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E02              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCC
GU227862         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCC
O03              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCC
F37              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCC
D06              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCC
BKM              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
FJ972612         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
MO1              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
NO1              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
JX010276         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
JX010171         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
O11              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
O05              TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
JX010173         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
JX010176         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCTGATGTAGGCC
EU371022         TCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACAGCCGATGTAGGCC
A13              TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
A15              TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
E15              TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
C04              TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
JQ948267         TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
JQ948276         TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
JQ948291         TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
STP22            TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
TH5              TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
JQ948288         TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCACACGTGGGCC
JQ005776         TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCACGGCAGACGTGGGCC
GQ485607         TCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGGCTTCCGTAGGCC
P2P1D            TCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGGCTTCCGTAGGCC
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B16               CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
E02               CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
GU227862          CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
O03               CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
F37               CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
D06               CTTAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACATT
BKM               CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
FJ972612          CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
MO1               CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
NO1               CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
JX010276          CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
JX010171          CTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTTT
O11               CTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTTT
O05               CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
JX010173          CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
JX010176          CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
EU371022          CTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
A13               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
A15               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
E15               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
F59               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
C04               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
JQ948267          CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
JQ948276          CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
JQ948291          CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
STP22             CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
TH5               CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
JQ948288          CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
JQ005776          CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC-TA
GQ485607          CCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACATA
P2P1D             CCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACATA
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E02               TCGTCTCGCATTGGGATTCGGAGGGACTCTAGCCGTAAAACCCCCA-
GU227862          TCGTCTCGCATTGGGATTCGGAGGGACTCTAGCCGTAAAACCCCCA-
O03               TCGTCTCGCATTGGGATTCGGAGGGACTCTAGCCGTAAAACCCCCA-
F37               TCGTCTCGCATTGGGATTCGGAGGGACTCTAGCCGTAAAACCCCCA-
D06               TCGTCTCGCATTGGGATTCGGAGGGACTCTGGCCGTAAAACCCCCA-
BKM               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
FJ972612          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
MO1               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
NO1               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
JX010276          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
JX010171          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCC--
O11               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
O05               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
JX010173          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
JX010176          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
EU371022          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
A13               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
A15               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
E15               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
F59               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
C04               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
JQ948267          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
JQ948276          ACGTCTCGCACTGGGATTCGGAGGGACTCTTGCCGTAAAACCCCCA-
JQ948291          ACGTCTCGCACTGGGATTCGGAGGGACTCTTGCCGTAAAACCCCCA-
STP22             ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
TH5               ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
JQ948288          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC-
JQ005776          ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCA-
GQ485607          CCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTAAAACCCCCC-
P2P1D             CCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTAAAACCCCCC-
```

Appendix 5.0 Modeltest (Posada and Crandall, 1998) output for evolutionary model selection of ITS1-5.8S-ITS2 alignment using the corrected Akaike Information Criterion (AICc) (Akaike, 1974) without using branch lengths parameters.

```
------------------------------------------------------------- *
* *        CORRECTED AKAIKE INFORMATION CRITERION (AICc)        * *
* ------------------------------------------------------------
Settings:   Sample size = 495
 Model selected:     Model = TrN+G
   partition = 010020
   -lnL = 1333.0692
   K = 66
   freqA = 0.2267
   freqC = 0.3031
   freqG = 0.2428
   freqT = 0.2275
   R(a) [AC] =  1.0000
   R(b) [AG] =  1.0821
   R(c) [AT] =  1.0000
   R(d) [CG] =  1.0000
   R(e) [CT] =  4.2018
   R(f) [GT] =  1.0000
   gamma shape = 0.2210
--
PAUP* Commands Block:  If you want to load the selected model and
associated estimates in PAUP*,  attach the next block of commands
after the data in your PAUP file:
[!
Likelihood settings from best-fit model (TrN+G) selected by AICc
with jModeltest 0.1.1 on Thu Aug 29 20:22:46 SGT 2013]
BEGIN PAUP;
Lset base=(0.2267 0.3031 0.2428 0.2275) nst=6  rmat=(1.0000 1.0821
1.0000 1.0000 4.2018 1.0000) rates=gamma shape=0.2210 ncat=4 pinvar=0;
END;
--

* AICc MODEL SELECTION : Selection uncertainty
Model             -lnL    K         AICc     delta      weight
cumWeight
-----------------------------------------------------------
TrN+G       1333.0692   66   2818.8020     0.0000      0.2245    0.2245
TIM1+G      1332.1604   67   2819.6603     0.8583      0.1461    0.3706
TrNef+G     1337.7462   63   2820.2024     1.4004      0.1114    0.4820
TIM3ef+G    1336.4621   64   2820.2730     1.4710      0.1076    0.5896
TIM3+G      1332.6158   67   2820.5712     1.7692      0.0927    0.6823
TIM1ef+G    1336.7084   64   2820.7657     1.9637      0.0841    0.7664
TIM2+G      1332.9514   67   2821.2423     2.4403      0.0663    0.8327
TIM2ef+G    1337.7120   64   2822.7728     3.9707      0.0308    0.8635
SYM+G       1335.2325   66   2823.1285     4.3265      0.0258    0.8893
GTR+G       1331.3713   69   2823.4721     4.6701      0.0217    0.9110
TrN+I+G     1334.2165   67   2823.7725     4.9705      0.0187    0.9297
TIM2+I+G    1332.9518   68   2823.9317     5.1297      0.0173    0.9470
TIM1+I+G    1333.2994   68   2824.6269     5.8249      0.0122    0.9592
TrNef+I+G   1338.9379   64   2825.2246     6.4226      0.0090    0.9682
TIM3ef+I+G  1337.6611   65   2825.3221     6.5201      0.0086    0.9768
TIM3+I+G    1333.7542   68   2825.5365     6.7345      0.0077    0.9846
TIM1ef+I+G  1337.8737   65   2825.7475     6.9455      0.0070    0.9915
TIM2ef+I+G  1338.9283   65   2827.8567     9.0547      0.0024    0.9940
SYM+I+G     1336.4054   67   2828.1503     9.3483      0.0021    0.9961

GTR+I+G     1332.4693   70   2828.3820     9.5799      0.0019    0.9979
HKY+G       1340.2149   65   2830.4299    11.6278      0.0007    0.9986
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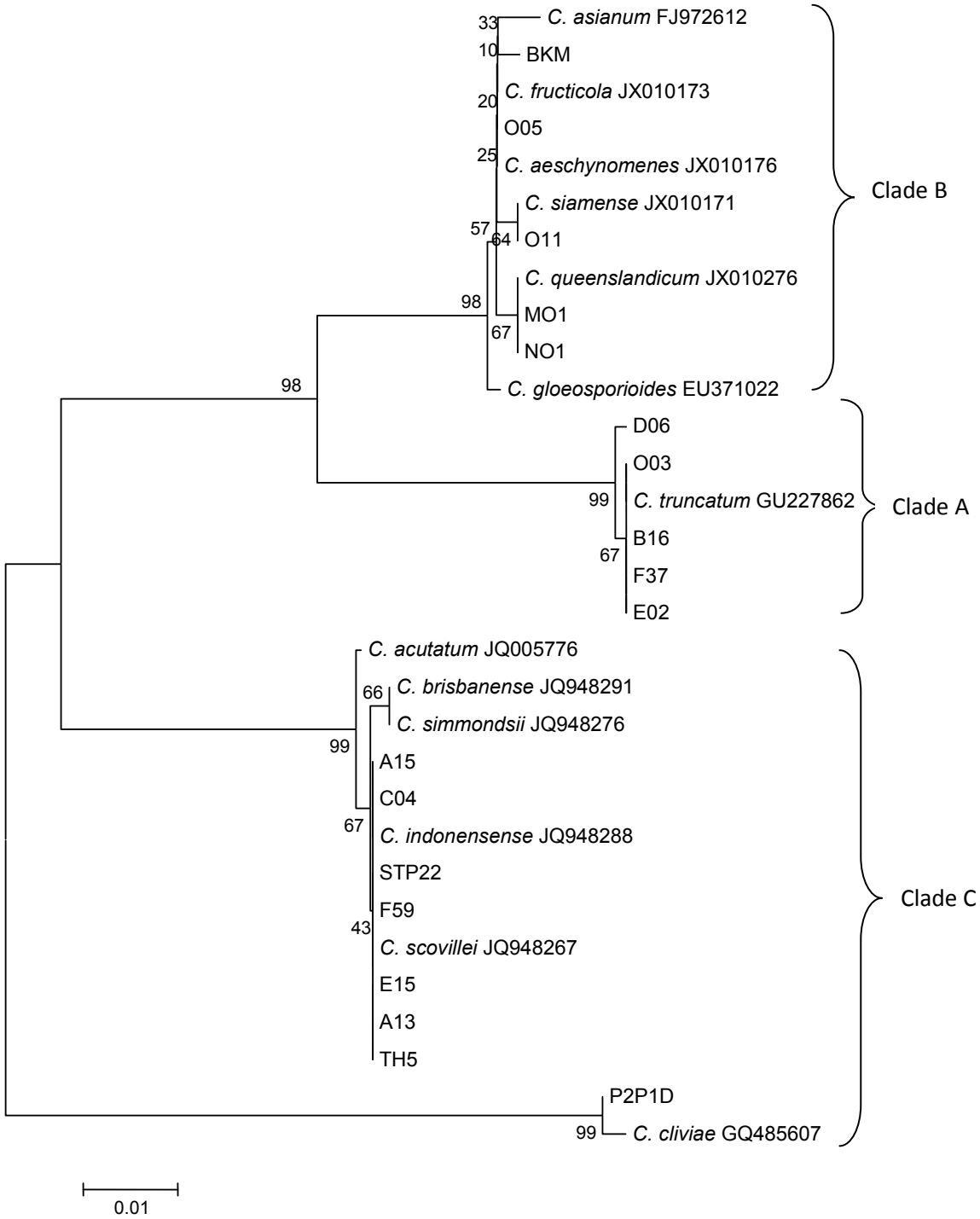
TPM1uf+G	1339.2427	66	2831.1491	12.3470	0.0005	0.9991
TPM3uf+G	1339.6587	66	2831.9810	13.1790	0.0003	0.9994
TPM2uf+G	1340.0232	66	2832.7099	13.9079	0.0002	0.9996
TVM+G	1338.2399	68	2834.5079	15.7059	8.72e-005	0.9997
TPM2uf+I+G	1340.0236	67	2835.3868	16.5848	5.62e-005	0.9997
HKY+I+G	1341.3884	66	2835.4404	16.6384	5.47e-005	0.9998
TPM1uf+I+G	1340.4039	67	2836.1474	17.3454	3.84e-005	0.9998
K80+G	1347.3756	62	2836.8346	18.0326	2.73e-005	0.9999
TPM3uf+I+G	1340.8296	67	2836.9988	18.1968	2.51e-005	0.9999
TPM3+G	1346.1453	63	2837.0006	18.1986	2.51e-005	0.9999
TPM1+G	1346.2339	63	2837.1778	18.3758	2.30e-005	0.9999
TPM2+G	1347.3257	63	2839.3614	20.5594	7.70e-006	0.9999
TrN+I	1343.4317	66	2839.5269	20.7249	7.09e-006	0.9999
TVM+I+G	1339.4153	69	2839.5600	20.7580	6.98e-006	1.0000
TIM3ef+I	1346.1124	64	2839.5735	20.7715	6.93e-006	1.0000
TVMef+G	1344.7906	65	2839.5813	20.7792	6.90e-006	1.0000
TrNef+I	1347.5568	63	2839.8235	21.0215	6.11e-006	1.0000
TIM1+I	1342.5041	67	2840.3479	21.5459	4.70e-006	1.0000
TIM1ef+I	1346.5359	64	2840.4207	21.6187	4.54e-006	1.0000
TIM3+I	1342.7906	67	2840.9208	22.1188	3.53e-006	1.0000
K80+I+G	1348.5514	63	2841.8129	23.0108	2.26e-006	1.0000
TPM3+I+G	1347.3415	64	2842.0318	23.2298	2.03e-006	1.0000
TPM1+I+G	1347.4035	64	2842.1558	23.3537	1.91e-006	1.0000
TIM2+I	1343.4300	67	2842.1997	23.3977	1.86e-006	1.0000
TIM2ef+I	1347.5516	64	2842.4520	23.6500	1.64e-006	1.0000
SYM+I	1344.9528	66	2842.5692	23.7671	1.55e-006	1.0000
GTR+I	1341.6697	69	2844.0688	25.2668	7.32e-007	1.0000
TPM2+I+G	1348.5376	64	2844.4241	25.6221	6.13e-007	1.0000
TVMef+I+G	1345.9922	66	2844.6479	25.8458	5.48e-007	1.0000
F81+G	1349.9274	64	2847.2037	28.4017	1.53e-007	1.0000
HKY+I	1350.0334	65	2850.0668	31.2648	3.65e-008	1.0000
TrN	1350.0708	65	2850.1415	31.3395	3.51e-008	1.0000
TIM3ef	1352.7699	63	2850.2497	31.4477	3.33e-008	1.0000
TrNef	1354.1736	62	2850.4306	31.6286	3.04e-008	1.0000
TPM1uf+I	1349.0697	66	2850.8030	32.0010	2.52e-008	1.0000
TIM1	1349.1459	66	2850.9553	32.1533	2.34e-008	1.0000
TIM1ef	1353.1872	63	2851.0844	32.2824	2.19e-008	1.0000
TIM3	1349.2929	66	2851.2493	32.4473	2.02e-008	1.0000
TPM3uf+I	1349.3036	66	2851.2708	32.4688	2.00e-008	1.0000
F81+I+G	1351.1234	65	2852.2468	33.4448	1.23e-008	1.0000
TPM2uf+I	1350.0281	66	2852.7197	33.9177	9.68e-009	1.0000
TIM2	1350.0689	66	2852.8014	33.9993	9.30e-009	1.0000
TIM2ef	1354.1375	63	2852.9850	34.1830	8.48e-009	1.0000
SYM	1351.6737	65	2853.3475	34.5455	7.07e-009	1.0000
JC+G	1357.1421	61	2853.7531	34.9511	5.78e-009	1.0000
TVM+I	1348.1384	68	2854.3050	35.5030	4.38e-009	1.0000
GTR	1348.2359	68	2854.4999	35.6979	3.98e-009	1.0000
TPM3+I	1355.3407	63	2855.3913	36.5893	2.55e-009	1.0000
K80+I	1356.7872	62	2855.6578	36.8558	2.23e-009	1.0000
TPM1+I	1355.7246	63	2856.1592	37.3572	1.73e-009	1.0000
TPM2+I	1356.7833	63	2858.2766	39.4746	6.02e-010	1.0000
TVMef+I	1354.1503	65	2858.3006	39.4985	5.94e-010	1.0000
JC+I+G	1358.3434	62	2858.7701	39.9681	4.70e-010	1.0000
HKY	1356.3603	64	2860.0695	41.2675	2.45e-010	1.0000
TPM1uf	1355.4059	65	2860.8117	42.0097	1.69e-010	1.0000
TPM3uf	1355.5261	65	2861.0523	42.2503	1.50e-010	1.0000
TPM2uf	1356.3596	65	2862.7192	43.9172	6.53e-011	1.0000
TVM	1354.4394	67	2864.2184	45.4164	3.08e-011	1.0000
TPM3	1361.4512	62	2864.9858	46.1838	2.10e-011	1.0000
K80	1362.8973	61	2865.2635	46.4615	1.83e-011	1.0000
TPM1	1361.8779	62	2865.8392	47.0371	1.37e-011	1.0000
F81+I	1359.3932	64	2866.1353	47.3332	1.18e-011	1.0000

TPM2	1362.8577	62	2867.7988	48.9967	5.15e-012	1.0000
TVMef	1360.3295	64	2868.0079	49.2059	4.64e-012	1.0000
JC+I	1366.1121	61	2871.6930	52.8910	7.35e-013	1.0000
F81	1365.6137	63	2875.9373	57.1353	8.80e-014	1.0000
JC	1372.0763	60	2881.0189	62.2168	6.93e-015	1.0000

-lnL: negative log likelihod K: number of estimated parameters
AICc: Corrected Akaike Information Criterion delta: AICc difference
weight: AICc weight cumWeight: cumulative AICc weight
Model selection results also available at the "Model > Show model table" menu

Appendix 6.0 Bootstrap distance tree for ITS-5.8S-ITS2 region generated using Neighbour-joining analysis. The number at the branches indicates the confidence values obtained from bootstrap analysis using 1000 replicates.



Appendix 7.0 Bootstrap maximum parsimony (MP) tree of the ITS-5.8S-ITS2 region. The tree is one of the most parsimonious trees generated from heuristic search of the parsimony informative sites. The number at the branches indicates the confidence values obtained from bootstrap analysis using 1000 replicates.