EXPLORING FUNGAL SPECIES BOUNDARIES USING MORPHOLOGY, DIVERGENCE TIMES, COALESCENT AND DISTANCE BASED METHODS

CHITRABHANU SHARMA BHUNJUN

DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY
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ABSTRACT

Fungi are an essential component of any ecosystem, but they can also cause mild and severe plant diseases. Plant diseases are caused by a wide array of fungal groups that affect a diverse range of hosts with different tissue specificities. Species delimitation is the process by which species boundaries are determined and new species are discovered. This is a major topic in modern systematics with increasing amount of molecular data available on public databases and the development of new methods for species delimitation. Fungi were previously named based only on morphology and, in many cases, host association, which has led to superfluous species names and synonyms. Morphology-based identification represents an important method for genus level identification and molecular data are important to accurately identify species. Accurate identification of fungal pathogens is vital as the scientific name links the knowledge concerning a species including the biology, host range, distribution, and potential risk of the pathogen, which are vital for effective control measures. Thus, in the modern era, a polyphasic approach is recommended when identifying fungal pathogens. It is also important to determine if the organism is capable of causing host damage, which usually relies on the application of Koch's postulates for fungal plant pathogens. The importance and the challenges of applying Koch's postulates are discussed. Bradford Hill criteria, which are generally used in

establishing the cause of human disease, are briefly introduced. We provided guidelines for pathogenicity testing based on the implementation of modified Koch's postulates incorporating biological gradient, consistency, and plausibility criteria from Bradford Hill. We provided a set of protocols for fungal pathogenicity testing along with a severity score guide, which takes into consideration the depth of lesions. The application of a standard protocol for fungal pathogenicity testing and disease assessment in plants will enable inter-studies comparison, thus improving accuracy. When introducing novel plant pathogenic fungal species without proving the taxon is the causal agent using Koch's postulates, we advise the use of the term *associated with the* "*disease symptoms*" *of* "*the host plant*".

This study also emphasizes on the application of different molecular-based methods for species delineation in two important pathogenic genera, *Bipolaris* and *Colletotrichum*. *Bipolaris* species are important plant pathogens with a worldwide distribution in tropical and temperate environments. Species recognition in *Bipolaris* has been problematic due to a lack of molecular data from ex-type cultures, the use of few gene regions for species resolution and overlapping morphological characters. In this study, we evaluated the efficiency of different DNA barcodes in species delimitation in *Bipolaris* by phylogenetic analyses, Automatic Barcode Gap Discovery and Objective Clustering. GAPDH is determined to be the best single marker for the genus. These approaches are used to clarify the taxonomic placement of all sequences currently named as *Bipolaris* in GenBank based on ITS and GAPDH gene sequence data. In checking various publications, we found that the majority of new host records published in the Plant Disease journal between 2010 and 2019 were based on blast searches of the ITS sequences and up to 82% of those records could be erroneous. Therefore, ITS Blast searches of GenBank to name species is not recommended. Editorial boards of journals and reviewers of new record papers should be aware of this problem. In naming *Bipolaris* species, whether new or known, it is recommended to perform phylogenetic analyses based on GAPDH using the correct

taxon sampling for accurate results and the clade should have reliable statistical support. At least two additional species are represented by molecular data in GenBank and we provide an updated taxonomic revision of *Bipolaris*. We accepted 45 species in *Bipolaris* and notes are provided for all the species including hosts and geographic distribution.

Colletotrichum is one of the most important plant pathogenic genera that is responsible for numerous diseases which can have a profound impact on the agricultural sector. Species delineation is difficult due to a lack of distinctive phenotypic variation. Therefore, in this study three different genomic approaches based on phylogenetic, evolutionary and coalescent-based methods were applied to establish robust species boundaries. The efficiency of five different DNA barcodes was determined for species delineation. The ITS region can resolve the generic placement of taxa up to the species complex level. The GAPDH and TUB2 markers are determined to be the most informative for most complexes. However, no single marker could discriminate between species in all complexes, therefore different molecular approaches based on multi-locus datasets is recommended. This is the first study to provide an estimated divergence time for all species complexes in *Colletotrichum*. The estimated divergence time for species complexes ranged between 4.8 to 32.2 MYA. Based on congruent results among different molecular approaches, a new species complex, the *Colletotrichum-agaves* complex was introduced. This complex consists of five taxa which are characterised by the presence of straight or slightly curved conidia with obtuse apices. This study shows that coalescent approaches and multi-locus phylogeny are crucial to establish species boundaries in *Colletotrichum*. The taxonomic placement of three singleton taxa *Colletotrichum axonopodi, C. cariniferi* and *C. parallelophorum* is revised. We accepted 248 species and provided recommendations regarding species boundaries in the graminicola-caudatum complex.

This study also resulted in the introduction of several novel taxa from several hosts. A new species, *Colletotrichum artocarpicola,* collected on *Artocarpus heterophyllus* from Chiang Rai, Thailand, was introduced using both morphological and molecular approaches. Combined phylogenetic analysis of ITS, GAPDH, CHS-1, ACT and TUB2 sequence data demonstrated that *Colletotrichum artocarpicola* is a distinct species within the gloeosporioides species complex. The new species is illustrated and compared with related taxa, and evidence of its pathogenicity was provided. A novel genus, *Anastomitrabeculia,* was also introduced for a distinct species, *Anastomitrabeculia didymospora*, collected as a saprobe on dead bamboo culms from a freshwater stream in Thailand. *Anastomitrabeculia* is distinct in its trabeculate pseudoparaphyses and ascospores with longitudinally striate wall ornamentation. A new family, *Anastomitrabeculiaceae,* is introduced to accommodate *Anastomitrabeculia*. *Anastomitrabeculiaceae* formed an independent lineage basal to *Halojulellaceae* in *Pleosporales* and it is closely related to *Neohendersoniaceae* based on phylogenetic analyses of a combined LSU, SSU and TEF1α dataset. In addition, divergence time estimates provided further support for the establishment of *Anastomitrabeculiaceae.* The family diverged around 84 million years ago (MYA) during the Cretaceous period, which supports the establishment of the new family. The crown and stem age of *Anastomitrabeculiaceae* was also compared to morphologically similar pleosporalean families. This study also introduced a new species from leaf litter samples. Leaf litter is an important component of the ecosystem as it is a major source of organic material. *Phyllosticta doitungensis* was introduced from leaf litter samples of *Dasymaschalon obtusipetalum.* The host, *Dasymaschalon obtusipetalum* is widely distributed in Asia and has been used as traditional medicine. Morphological descriptions and illustrations of the novel microfungi was also provided.

Fungi play vital roles in the ecosystems as endophytes, pathogens and saprobes. The current estimate of fungal diversity is highly uncertain, ranging from

1.5 to 12 million, but only around 150,000 species have been named and classified to date. Since the introduction of DNA based methods, the number of newly described fungal taxa has increased from approximately 1,000 to around 2,000 yearly. This demonstrates the importance of DNA based methods to identify and distinguish species, especially morphologically similar taxa. Many novel species from recent studies have been found in historically understudied regions and habitats, but these still represent only a small percentage of the estimated species. In this study, we estimate the number of taxa in the top 30 most speciose genera as listed in Species Fungorum. The genera that are treated herein are *Cercospora, Diaporthe, Meliola, Passalora, Phyllachora*, *Phyllosticta, Pseudocercospora, Ramularia* (ascomycetes) and *Cortinarius*, *Entoloma*, *Inocybe*, *Marasmius*, *Psathyrella*, *Puccinia, Russula, Uromyces* (basidiomycetes). We discuss why these genera have some of the largest number of species.

Keywords: BEAST, Disease Severity, DNA Barcoding, Dothideomycetes, Fungal Diversity, General Mixed Yule Coalescent Method, Host-specificity, Image Analysis, Integrative Taxonomy, Multi-rate Poisson Tree Process, Pathogenicity, Plant Disease Assessment

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ABBREVIATIONS AND SYMBOLS

ABGD	automatic barcode gap discovery
ACT	actin
bp	base pair
CHS-1	chitin synthase 1
cm	centimetre
ESS	effective sample size
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCPSR	genealogical concordance phylogenetic species recognition
GMYC	generalized mixed Yule-coalescent
GTR	general time reversible model
ITS	internal transcribed spacer region
LSU	nuclear ribosomal large subunit 28S rRNA gene
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
Mya	million years ago
ng	nanogram
PDA	potato dextrose agar
PHI	pairwise homoplasy index
PTP	Poisson tree processes
RPB2	RNA polymerase II second largest subunit
SSU	nuclear ribosomal small subunit 18S rRNA gene
TEF1α	translation elongation factor 1-alpha gene
TUB2	β-tubulin

ABBREVIATIONS AND SYMBOLS (continued)

°C	degree Celsius
µl	microlitre
µM	micromole
α	alpha
β	beta

CHAPTER 1

INTRODUCTION

1.1 Introduction to Exploring Fungal Species Boundaries Using Polyphasic Approaches

Fungi are an important part of the ecosystem due to their ability to produce a range of extracellular enzymes that break down cellulose, hemicellulose and lignin [1]. This decomposition process releases key plant nutrients back into the soil and without nutrient cycling, life on earth would not be possible. Fungi are an ancient, diverse and heterogeneous group of organisms [1]. They can be found in a wide range of habitats, ranging from the stratosphere [2] to the bottom of the dead sea [3], from the Antarctic glaciers [4] to torrid deserts [5] and from the gut of flies [6] to deep oceanic sediments [7]. Fungi are an important group of organisms with potential uses in biotechnology [8]. They can produce secondary metabolites with a diverse range of biological activities that can be targeted for drug discovery [9]. Fungi can also cause several serious human, animal and plant diseases. The accurate identification at the species level is therefore vital for its application in industrial, agrochemical, or pharmaceutical sector. Fungi are one of the most species-rich group [10], however, only 150,000 species have been named and classified so far [1]. This is much lower than the estimated 1.5 million by Hawksworth [11], 5.1 million by Blackwell [12] and 12 million by Wu et al. [13]. Fungi can exhibit several different lifestyles including endophytes, biotrophs, hemi–biotrophs, necrotrophs and saprotrophs.

Species delineation remains a significant challenge and several species concepts have been applied in an attempt to provide clarification at the species level. In the past, species delineation was mainly based on morphological similarity [14], however, species delimitation and identification based on morphology alone is difficult due to overlapping morphological characters in several genera. The

combination of morphology and molecular data have provided a more reliable system of classification in fungal taxonomy [15–21] however, there is still much discordance at lower taxonomic levels. Molecular data have provided a better understanding in circumscribing fungal species, but most studies have only focused on the phylogenetic species concept.

This study mainly focused on the identification of fungi by delimiting species boundaries which often require a range of independent dataset and analytical approaches. Therefore, this study applied different molecular-based approaches for species delineation in selected genera.

Note Methods used for species delimitation.

Figure 1.1 The selected approaches used in this study

1.2 Research Background of Applying Species Delineation Methods

Species delimitation and determining species boundaries are crucial to understand the evolution of organisms and how best to manage biodiversity [22]. The effects of global warming to biodiversity has created a need for more effective ways to discover species. Novel algorithmic approaches for analyzing sequence data combined with rapidly expanding DNA barcode libraries provide a potential solution [23]. As such, robust progress has been made towards resolving the phylogenetic position of fungal species. Species are fundamental units in biological research and often few molecular based methods are used to corroborate identification and delineation of new fungal taxa [24]. Fungi can be correctly classified by careful observation of characters at different levels, adequate taxon sampling and the application of robust methods of analysis.

The phylogenetic species concept in fungi has two broadly defined forms, the strict genealogic concordance (SGC) and the coalescent-based species delimitation (CBD). The most widely used coalescent methods, the generalized mixed Yule-coalescent (GMYC) and Poisson tree processes (PTP) were designed for the analysis of single-locus data but are often applied to concatenations of multi-locus data [25]. The GMYC uses a likelihood approach to identify the boundary between a Yule speciation process and intraspecific coalescence. The Bayesian multispecies (BPP) coalescent approach explicitly models the evolution of multi-locus data. The performance of GMYC, PTP and BPP were compared using dataset generated by simulation under various speciation scenarios. The number of loci and sample size per species were found to affect the determination of the correct guide trees based on given priors [23]. The BPP is widely accepted as it has lower rates of species overestimation and underestimation and is generally robust to various potential confounding factors except for high levels of gene flow [26]. The single-threshold GMYC and the best strategy identified in PTP generally perform well for scenarios involving more than a single putative species when gene flow is absent, but PTP outperforms GMYC when fewer species are involved [27]. Both methods are more sensitive than BPP to the effects of gene flow and potential confounding factors.

Therefore, to reflect a natural classification of species, it is important to use a correct taxon sampling with several molecular-based approaches such as phylogenetic analysis coupled with coalescent-based species delimitation (Beast, PTP, GMYC, and BPP) or distance-based DNA barcoding (ABGD, Objective clustering) [28]. The systematics of fungi were addressed by using these methods within an integrative taxonomic framework.

Species are diagnosed as a cluster of individuals that are sufficiently differentiated from other relative clusters revealed by DNA sequences [29]. The estimation of species diversity in fungal communities is based either on species counts or on the assignment of operational taxonomic data. Despite the large number of taxa on GenBank, there is a lack of studies that estimates the number of new species that could be present in some of the speciose genera that are listed on Species Fungorum (2021). Some of the most speciose genera listed in Species Fungorum (2021) includes *Meliola* with over 2,600 epithets, the basidiomycete genera *Puccinia* with over 4,000 epithets. A large number of species epithets in these genera is partly because species have been introduced based on host association or morphology [30]. There are also a large number of epithets listed in several phytopathogenic genera such as *Colletotrichum* with approximately 935 species and *Fusarium* with 1,676 epithets in Index Fungorum (2021). However, the number of species accepted in these genera are far lower than these numbers which shows the need to estimate the correct number of epithets based on data available.

1.3 Clarification of Species Boundaries in Selected Genera Based on a Polyphasic Classification

In this study, several molecular approaches for species delineation were applied to *Colletotrichum* and *Bipolaris*. *Bipolaris* species are important plant pathogens with a worldwide distribution in tropical and temperate environments. Species recognition in *Bipolaris* has been problematic due to a lack of molecular data from ex-type cultures, the use of few gene regions for species resolution and overlapping morphological characters. *Colletotrichum* species are endophytes, saprobes and pathogens and several species have been known to cause human infections. Morphology based delimitation of *Colletotrichum* species is problematic due to overlapping morphological characters in asexual morphs and the lack of sexual morphs. There are also several species that were named based on their hosts' specificity. The phylogenetic species concept was applied to check if species placement differed between the different methods (ML, MP and Bayesian Inference). Molecular based approaches such as Automatic Barcode Gap Discovery (ABGD) and Objective Clustering were also used for species delineation. ABGD is an analytical tool that detects a barcode gap in the distribution of pairwise genetic distances within the dataset [23, 31]. Objective clustering is based on pairwise distances to determine the intra– and inter–specific genetic distances to cluster sequences [32–33].

Plant litter can be described as a collection of fallen leaves, twigs, seeds, flowers and other woody debris [34–35]. Plant litter accumulation alters the surrounding environment by intercepting light, retaining moisture and maintaining soil temperature [36–38]. The process of plant litter decomposition is vital as it releases nutrients and carbon dioxide to the atmosphere. There are several studies that have focused on the diversity of fungi on plant litter, however, these studies have introduced novel species based only on phylogenetic analyses [39–41]. In this study, microfungi were introduced from the root of *Artocarpus heterophyllus,* leaf litter samples of *Dasymaschalon obtusipetalum* and from dead bamboo culms based on several molecular based approaches.

1.4 Research Objectives

1.4.1 Apply a number of molecular-based approaches for species delineation of specific genera and study their phylogenetic relationships.

1.4.2 This study provides guidelines for pathogenicity testing based on Koch's postulates.

1.4.3 This project catalogues fungal species and provides morphological descriptions and illustrations of micro-fungi associated with plant litter.

1.4.4 Generate molecular data of saprobes associated with plant litter in Thailand.

1.5 Research Contents

This thesis is divided into eight chapters.

Chapter 1 comprises of a general introduction to the research background of exploring fungal species boundaries using morphology, divergence times, coalescent and distance-based methods.

Chapter 2 presents details on the importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's postulates.

Chapter 3 introduces a polyphasic approach to delineate species in *Bipolaris.*

Chapter 4 investigates species boundaries in *Colletotrichum.*

Chapter 5 introduces multigene phylogenetic characterization of C*olletotrichum artocarpicola sp. nov.* from *Artocarpus heterophyllus* in northern Thailand.

Chapter 6 introduces the application of integrating different lines of evidence to establish a novel ascomycete genus and family (*Anastomitrabeculia, Anastomitrabeculiaceae*) in *Pleosporales.*

Chapter 7 presents the numbers of fungi: are the most speciose genera truly diverse?

Chapter 8 provides the overall conclusion of the thesis, the advances made in this study and suggestions for future work.

CHAPTER 2

IMPORTANCE OF MOLECULAR DATA TO IDENTIFY FUNGAL PLANT PATHOGENS AND GUIDELINES FOR PATHOGENICITY TESTING BASED ON KOCH'S POSTULATES[1]

Abstract

Fungi are an essential component of any ecosystem, but they can also cause mild and severe plant diseases. Plant diseases are caused by a wide array of fungal groups that affect a diverse range of hosts with different tissue specificities. Fungi were previously named based only on morphology and, in many cases, host association, which has led to superfluous species names and synonyms. Morphology-based identification represents an important method for genus level identification and molecular data are important to accurately identify species. Accurate identification of fungal pathogens is vital as the scientific name links the knowledge concerning a species including the biology, host range, distribution, and potential risk of the pathogen, which are vital for effective control measures. Thus, in the modern era, a polyphasic approach is recommended when identifying fungal pathogens. It is also important to determine if the organism is capable of causing host damage, which usually relies on the application of Koch's postulates for fungal plant pathogens. The importance and the challenges of applying Koch's postulates are discussed. Bradford Hill criteria, which are generally used in establishing the cause of human disease, [1]

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I declare that my contribution to this chapter was 95%.

are briefly introduced. We provide guidelines for pathogenicity testing based on the implementation of modified Koch's postulates incorporating biological gradient, consistency, and plausibility criteria from Bradford Hill. We provide a set of protocols for fungal pathogenicity testing along with a severity score guide, which takes into consideration the depth of lesions. The application of a standard protocol for fungal pathogenicity testing and disease assessment in plants will enable inter-studies comparison, thus improving accuracy. When introducing novel plant pathogenic fungal species without proving the taxon is the causal agent using Koch's postulates, we advise the use of the term *associated with the* "*disease symptoms*" *of* "*the host plant*". Where possible, details of disease symptoms should be clearly articulated.

Keywords: Disease Severity, Image Analysis, Pathogenicity, Phylogeny, Plant Disease Assessment

2.1 Introduction

Animals and plants live in close contact with innumerable microorganisms, but only a small percentage can cause disease [42–43]. Fungi exhibit different types of associations with plants ranging from mutualism to parasitism [44]. They are vital in nutrient cycling which releases key plant nutrients into the soil [45]. As plant pathogens, they can cause significant damage in agriculture and forestry [46]. It was assumed that pathogenic microorganisms were fundamentally different from non-pathogenic microorganisms as they possess certain properties that are responsible for the pathogenic potential [47]. This led to the classification of microorganisms based on their pathogenic potential. This pathogen-centred view cannot be applied to certain organisms whose pathogenicity is dependent on the characteristics of the host rather than the microorganism [47]. *Candida albicans* and *Staphylococcus epidermitis*, for example, can cause diseases primarily in patients with impaired host defense or altered microbiota, whereas some microorganisms can be pathogens in normal individuals as well as those with impaired immunity [43]. Therefore, a pathogen cannot be defined based only on its ability to cause disease in hosts with impaired defense [47].

The definition of pathogen and host are intrinsically linked and host–pathogen interactions can be characterized based on whether they cause interruption of normal tissue structure or function of the host [43]. A pathogen can thus be described as an organism capable of causing host damage [48]. The host–pathogen interaction is one of the most complicated phenomena in biology [49]. Pathogens can be classified into biotrophs, necrotrophs, or hemibiotrophs based on their lifestyles [50]. Biotrophs such as rusts, powdery mildews, or *Peronospora* rely on living plant cells for nutrients [51]. Necrotrophs such as *Botrytis cinerea* or *Cochliobolus heterostrophus* rapidly kill the host tissues with toxins and cell wall degrading enzymes [51]. Hemibiotrophs such as *Pyricularia grisea* (*Magnaporthe grisea*), *Zymoseptoria tritici* (*Mycosphaerella graminicola*), and *Phytophthora infestans* have an initial period of biotrophy followed by the production of necrotrophic hyphae [51]. As a result, plants have evolved the ability to recognise and respond to pathogen, leading to rapid activation of defence responses [50].

Plant diseases can be caused by multiple fungal genera that affect diverse hosts with different tissue specificities involving a myriad of symptoms [49]. *Colletotrichum* species can cause anthracnose, foliar disease, rot, seedling blights, and post-bloom fruit drop in several hosts [52–54]. *Diaporthe* species are associated with root and fruit rots, dieback, stem cankers, leaf spots, and seed decay of a wide range of hosts [55]. *Pestalotiopsis* species can cause canker lesions, shoot dieback, leaf spots, needle blight, tip blight, grey blight, severe chlorosis, fruit rots, and post-harvest diseases in several economically important crops [46]. Fungal pathogens can cause devastating diseases that have a major financial impact and can also affect ornamental crops as well as the agricultural sector [52]. Some pathogens have led to the starvation of human populations such as *Phytophthora infestans*, which was responsible for the Irish potato famine, and *Bipolaris oryzae*, which was responsible for the 1943 Bengal famine in India [56]. It is estimated that fungal pathogens destroy one-third of all food crops annually [57]. Pathogenic fungi can also cause diseases in humans ranging from superficial infections to invasive infection that can lead to mortality [58]. It is estimated that fungal diseases are responsible for the death of over 1.6 million humans every year [58]. Therefore, a better understanding of pathogenicity, hosts, and methods of spread of fungal pathogens is vital for global biosecurity [52]. It is important to mention that

diseases can also have positive impact in ecosystems. The “Tree Disease Concepts” discuss that forests need a “healthy amount of disease”, as these disease-causing agents act as regulators, terminators, and resource recovery agents, allowing the forest to be sustained over time [59]. This concept points out that forest health is only affected when the impact of pathogens exceeds a level suitable for the sustainability of the system.

In this review, we stress the importance of accurately identifying fungal pathogenic species using a polyphasic approach. If the fungus is plant-associated and a likely pathogen, this needs to be proven. We review the history, importance, and challenges of applying Koch’s postulates. We discuss limitations related to pathogenicity testing of plants pathogens. We provide guidelines for pathogenicity testing based on Koch’s postulates modified with aspects of Bradford Hill criteria incorporating the biological gradient criterion, the consistency criterion, and the plausibility criterion. A set of protocols is provided as a guideline for pathogenicity testing of fungal pathogens on different parts of the plant along with a severity score guide.

2.2 Species Identification Using Molecular Data

When identifying the cause of a plant disease, such as leaf lesions, it is important to accurately identify the fungal species, as well as confirm if the organism is capable of causing damage to the host [46]. This is because several organisms are present as endophytes or epiphytes on plant surface and do not cause disease; therefore, it is vital to confirm that the isolate is responsible for the pathogenicity [60]. Endophytes can become latent plant pathogens under certain inherent or environmental conditions or they can be conditional pathogens, which causes diseases as the plant ages or under stress conditions [61]. If the fungus is sporulating on the leaf lesion, it is relatively easy to identify the species to genus via its morphology [62–66], and obtain an isolate of the taxon via single spore isolation [67]. The isolate can then be used for pathogenicity testing and multi-gene molecular approaches for species-level identification [67]. If there is no sporulating fungus on the lesion, however, a different approach is needed. In this approach, the plant tissues are surface sterilized to minimize contaminants and

the tissue is sectioned into small pieces that are placed on appropriate media, allowing for fungal growth [67]. This methodology is similar to the ones used for isolating endophytes [68–69], so it is not definitive that the causal agent will be isolated. This can result in the isolation of endophytes and pathogens, as this approach usually consists of the interphase or transitional area between healthy and infected tissue [60]. Therefore, it is important to inoculate the host with all the isolates to confirm that the correct isolate was obtained based on the observed symptoms. Sporulating isolates are identified to genus level via morphology and the isolates are identified to species level using multi-gene molecular approaches [67].

Pathogenic species were previously identified based only on morphology, which can lead to misidentification due to phenotypic plasticity [29]. Morphology-based identification was primarily based on spore characteristics [70]. This led to the implementation of different scientific names for species with different stages of sporing morphs, resulting in dual nomenclature [70]. Morphology-based identification resulted in recognizing morphospecies as a suite of indistinguishable taxa in several genera including *Phaeoacremonium*, which represent over 20 species based on molecular approaches [71]. Morphology-based identification is also a major limitation in several important pathogenic genera including *Bipolaris*, *Colletotrichum, Diaporthe*, and *Pythium*, as the species have overlapping morphology [52, 69, 72–73]. However, despite the limitations, morphology-based identification represents a fast way to identify isolates up to genus level [62–66].

Pathogenic species have also previously been identified based on their host association [74]. This resulted in morphologically similar fungi growing on different plant genera or plant species being given different scientific names [70]. The implementation of phylogenetic analyses provided a better understanding of species boundaries, which resulted in the implementation of using one name for one fungus [75]. Host-specificity alone cannot be used for species delineation, but it remains an important factor in biological control as a host-specific pathogen can have a limited distribution, whereas a taxon with a wide range of hosts is likely to be cosmopolitan [76]. However, our knowledge of host-specificity is limited as most studies have focused on economically important crops or ornamental crops [74]. A better understanding of host-specificity would require extensive sampling from different

geographic locations and extensive examination of different parts of hosts using a combination of direct and high throughput methods [77].

Phylogenetic analyses have been used extensively to clarify species boundaries in several fungal genera [76, 78]. However, there are several limitations associated with phylogenetic analyses for species identification [70]. Phylogenetic analyses do not account for hybridization events and horizontal gene transfer [70]. The internal transcribed spacer (ITS) region has been accepted as the universal barcode for fungi owing to the ease of amplification and its broad utility across the kingdom, but often, it can only be used for placement up to the genus level [79–80]. There are many species that have not been correctly identified in databases such as GenBank and there are also many unidentified species [72]. There is a lack of molecular data for several fungal species including reference sequences and some species only have ITS sequences, which hinders molecular-based approaches [69, 72]. There is also a lack of ex-type or authenticated sequences for several pathogenic genera [76]. In some genera, there is no agreement as to the barcode that should be used for species-level identification [69]. Many pathogens have high genetic variation within individual species owing to the species' historical dynamics, demography, and topography [81]. Pathogens have short generation times and large population sizes, which can lead to high levels of genetic variation owing to rapid adaptation to environmental factors and human-mediated factors [82]. The identification of species boundaries is thus important to better understand genetic variation in nature to develop sustainable control measures [83].

It is thus recommended to use a large taxon sampling based on multi-gene phylogeny of mitochondrial, nuclear, ribosomal, and protein-coding genes to accurately determine the phylogenetic relationships of taxa [70]. Novel taxa should ideally be introduced with more than one strain to provide insights into intraspecific phenotypic diversity and there should be reliable statistical support for the species relationship (at least 70% bootstrap or 0.90 posterior probabilities) [69]. It is also recommended to use different approaches including Bayesian inference, maximum likelihood, maximum parsimony coupled with automatic barcode gap discovery, coalescent-based methods, or the genealogical concordance phylogenetic species recognition to investigate species boundaries in pathogenic genera [69, 72, 76, 84]. These approaches have been used to derive a better understanding of species boundaries in many important genera including

Alternaria, *Bipolaris*, *Colletotrichum*, *Daldinia*, and *Diaporthe* [69, 72, 76, 79, 85–86]. Therefore, the application of a polyphasic approach based on morphology, ecology, and molecular-based approaches provides a solid framework for accurate species' delineation [69, 76, 79].

2.2.1 Koch's Postulates

Koch's postulates are usually used to determine if an isolate is capable of causing host damage in plants [87]. Robert Koch began his work on disease transmission in 1873 when there was widespread interest in the control and prevention of several diseases [87]. He established novel techniques for the identification, isolation, and visualization of bacteria, which he used to identify and trace the life cycle of *Bacillus anthracis* [87]. He conducted animal inoculation experiments with anthrax bacilli to demonstrate that they caused anthrax [87]. In 1882, he discovered the microorganism Tuberkelvirus (*Mycobacterium tuberculosis*), which was responsible for pulmonary tuberculosis [88]. Koch then embarked on isolating the microorganism and he was awarded the Nobel Prize in Medicine in 1905 for his investigations and discoveries concerning tuberculosis [88]. Koch's postulates were published in 1890 and they are often considered the first reliable method to establish whether a microorganism is the cause of disease [89]. These postulates were derived based on his work on infectious diseases such as anthrax and tuberculosis [89]. The postulates are commonly based on three basic concepts as follows: (a) the pathogen occurs in every occurrence of the disease; (b) the pathogen does not occur in other diseases as a fortuitous and non-pathogenic agent; and (c) after being fully isolated and repeatedly grown in pure culture, the pathogen can induce the disease again [90]. Koch concluded that, if all these conditions were met, then the occurrence of the pathogen in the disease could no longer be accidental [90]. The three concepts mentioned above are based on River's translation [91]. In the absence of an animal model, Koch relied on naturally occurring processes that introduced the contagion, which was demonstrated by his findings on cholera based on outbreaks in villages [87].

2.2.2 Limitations of Koch's Postulates

Koch recognized that his postulates had limitations as he stated that diseases such as anthrax, tetanus, and tuberculosis fulfilled all the postulates, while others did

not [92]. Therefore, they do not apply to all pathogens. The first postulate is difficult to apply when the occurrence of the pathogen precedes the development of the symptoms [87]. This postulate cannot be applied to diseases whereby the toxins produced by the pathogen exert their effect at a site distant from the site of multiplication, such as in diphtheria [92]. The second postulate does not account for the presence of asymptomatic carriers and the third postulate cannot be applied to pathogens that cannot be cultured on artificial media [87]. Koch's postulates often fail to accommodate the causal complexity characteristics of diseases [92]. Koch's first two postulates can be classified as causal specificity, in which a given type of effect can have only one type of cause and a given type of cause can have only one type of effect [87]. This mono-causal model does not apply to all diseases, which can have multiple causes or risk factors, and one cause can have different effects [87].

2.2.3 Bradford Hill Criteria

The Bradford Hill criteria have been extensively used for inferring causation and have been used to evaluate countless hypothesized relationships between occupational and environmental exposures and disease outcomes [93]. In 1965, Bradford Hill published nine viewpoints for evaluating traditional epidemiologic data, but emphasised that they were neither necessary nor sufficient for causation [93]. The nine viewpoints include the strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy [93]. Hill suggested that a strong association between exposure and disease is indicative of causation [94]. The consistency criterion is satisfied when there is a consistent association between two variables in different epidemiologic studies based on a variety of location, population, and methods [93]. The specificity criterion suggests that association is likely to be indicative of causation when they are specific, which means that the exposure causes only one disease [94]. The temporality criterion suggests that, for an exposure–disease relationship to be causal, the exposure must precede the onset of the disease [93]. According to the biological gradient criterion, the presence of a dose–response relationship supports the causal association between the exposure and the effect [95]. The plausibility criterion is satisfied if the relationship is consistent with the current knowledge regarding the aetiology and mechanism of disease [96]. The

coherence criterion is satisfied if the cause-and-effect relationship follows the current knowledge [93]. The experiment criterion suggests that the strongest support for causal inference is a decline in disease risk following an intervention or cessation of exposure [96]. The analogy criterion is satisfied when there is strong evidence of a causal relationship between a particular agent and a specific disease, then weaker evidence could be accepted that a similar agent may cause a similar disease [93].

2.2.4 Limitations of Bradford Hill Criteria

The major limitation of the Bradford Hill criteria is that there is no method to decide whether to assign a checkmark and how to make a final assessment [93]. Along with the magnitude of the association between exposure and disease, the statistical significance is also important to determine the potential causality, which could be affected by the underlying method and other factors [96]. The limitation of the specificity criterion is the single-factor relationship of causal inference, but most diseases can have multiple causes or risk factors [93]. The specificity criterion cannot be applied for most infectious or complex diseases in which multiple pathogens can produce the same set of symptoms or a single pathogen can produce a number of outcomes [97]. The biological gradient criterion could also be seen as limited as most dose–response curves vary between studies owing to characteristics of the population, exposure routes, and individual susceptibility [98]. Several diseases occur as a result of the interplay and balance between multiple contributing and intermediary factors [93]. Therefore, it is difficult to demonstrate the biological plausibility of a causal relationship. Diseases result from multifaceted exposures that follow complex progression pathways and cessation of exposure may not reverse or slow the progression of the disease [96]. This limits the application of the experiment criterion. It has been argued that the modern application of the analogy criterion is not satisfied from confirming a causal inference, but from proposing and testing mechanistic hypotheses [93].

2.2.5 Limitations of Artificial Inoculations

It is important to artificially inoculate plants to determine if an isolate is capable of causing host damage [99]. Artificial inoculations are often conducted on detached or whole plants under extreme conditions that can favour the success of the infection [99].

The success of the infection often depends on the inoculum density, especially when using mycelium plugs, which provides an energy source for the pathogen [100]. The use of conidial suspension is recommended as the infection and spread of pathogens is likely to occur via conidia or ascospores [101]. Therefore, using conidial suspensions is more likely to produce a similar result as in nature, but mycelium plugs are often used when it is difficult to produce spore suspensions with enough conidia [101]. The process of surface sterilization is another factor that should be performed properly to remove surface microorganisms to ensure reliable results [102]. Pathogenicity tests conducted on detached plant tissues may not be reliable owing to suppression of the host defence pathways [102]. Artificial host inoculation is not reliable to determine host range, but the data can be used as an indicator of the infection potential [102]. Artificial inoculations are affected by environmental and physiological factors including temperature, humidity, and plant maturity [103]. Therefore, it is important to control abiotic factors such as temperature and humidity as they can influence the virulence of several pathogens during artificial host inoculation [83]. Fungicolous fungi are a diverse group of organisms that are associated with other fungi as symbionts, mycoparasites, or saprotrophs [104]. They can cause serious diseases of cultivated edible and medicinal mushrooms, but artificial inoculations cannot be performed for these fungicolous fungi to determine their pathogenic potential [104].

2.2.6 The Wounded versus Non-Wound Method

Plants are continuously exposed to environmental stress, which can cause wounding [105]. Wounding provides nutrients to pathogens and facilitates their entry into the plant tissues [105]. Artificial inoculations are performed using wounded and non-wounded methods and the wounded method should only be used for pathogens that normally enter through damaged plant parts [105]. These include most species of *Botryosphaeriaceae*, which can enter plant tissue via wounds [106].

2.2.7 Limitation When Dealing with Root and Stem Pathogens

To assess the severity of roots' pathogens, the inoculated plants have to be carefully uprooted and examined at given sampling dates [107]. The severity of root pathogens can also be assessed based on features of the above-ground plant parts such as leaf mass and color [108]. The limitation of this method is that the relationship

between the root disease and the features of the shoots has to be established [108]. Most studies dealing with root and stem pathogen are carried out using the wounded method, which favors the pathogen [109]. Artificial inoculation should be performed on hosts of similar age as the initial infection. This is a major limitation when dealing with stem and root pathogens of mature plants (>5 years) owing to the difficulty of acquiring hosts of similar age for pathogenicity testing. This sometimes results in species isolated from mature stems being tested for pathogenicity on young, tender shoots [110].

2.2.8 Virulence versus Aggressiveness Assessment

There are two kinds of pathogenicity, virulence and aggressiveness [111]. Virulence is a qualitative component and describes the ability of a genetically homogenous strain to grow on a genetically homogenous host [112]. When races of pathogen and varieties of the host interact differentially, the races are said to differ in virulence. When they do not interact differentially, they are said to differ in aggressiveness. Aggressiveness is a quantitative component of pathogenicity [112]. Aggressiveness is defined as the capacity of a natural pathogen to infect a host species (not possessing major resistance genes) or susceptible genotype [113]. These two concepts of pathogenicity must be kept separate and there is no known evidence for a positive correlation between virulence and aggressiveness [111].

2.2.9 Limitation of Studies When Introducing New Fungal Pathogens

Studies introducing new fungal pathogenic species usually include data on pathogenicity testing [55, 114–115], but this has not been applied in all cases [116–117]. Inoculation of detached tissues allows disease assessment without destroying the whole plant, but it may not be reliable owing to the suppression of the host defence pathways [62]. Therefore, findings from detached inoculations should be regarded as preliminary findings that should be confirmed using the whole plant [62]. Another limitation of the detached method is difficulty in differentiating and describing the area of inoculation as the overall tissue changes colour after several days [114]. Different studies have used different scales in visual plant disease assessment, which is problematic for inter studies comparison. Some studies have used the nominal scale whereby the disease is graded as slight, moderate, or severe, but these scales have limited value as they are subjective and there is a lack of quantitative definition [108].

Some studies have used different tissues for pathogenicity testing to the one where the initial disease was observed [55]. Several studies have performed pathogenicity testing only in conditions of high humidity, which prevent the spores from drying out and enable germination, but it also favours the success of the infection [118]. There is also a lack of replication in some studies, which can lead to biased results [119]. There is a lack of cross pathogenicity testing in some studies, which is an important indicator of the infection potential [114].

2.2.10 Disease Assessment Based on Alternative Technologies

Disease assessment is important for the quantification of diseases and screening for resistance [108]. Inaccurate assessment of disease severity can lead to incorrect actions in disease management [108]. Visual assessment is one of the most widely used methods, but symptom severity assessed visually remains an estimate [120]. Owing to the limitations with visual assessments, several methods have been developed to detect and quantify diseases including image analysis and visible wavelength photography [119]. The main advantage of image analysis and visible wavelength photography is that they provide a non-destructive, non-invasive, and permanent record of the disease severity for future reference [119]. Image analysis has also been used to develop standard area diagrams that improve the accuracy and precision in visual assessment of disease severity [120]. Standard area diagrams have been developed for several important diseases including leaf spot of sunflower caused by *Alternaria*, rust of bean leaves caused by *Uromyces appendiculatus,* foliar disease in pyrethrum crops, and stem rust in seed crops of perennial ryegrass [121–124]. Standard area diagrams can also be generated using software by taking into consideration the leaf and disease symptoms, which allows its application to a range of diseases [120].

Visible wavelength photography has often been combined with image analysis to record, detect, or measure disease in plants [119]. It has also been used to study the host–pathogen interaction, especially disease resistance and pathogen aggressiveness [125–126]. The image of the diseased sample can be acquired using digital cameras or flatbed scanners and the image is edited using image processing software such as Adobe Photoshop (Adobe Systems, United States) [119]. Pixel colour is defined by the hue, intensity, and saturation, which are often used to separate healthy from diseased areas

[127]. The number of pixels in the diseased versus healthy area is then used to calculate the percentage of the diseased area or lesion counts [128]. The differentiation of diseased versus healthy area can be a source of subjectivity, and it can also be difficult to distinguish between multiple diseases in the images [120]. The accuracy of measurements from image analysis can also be affected by several factors including focus, shadow, the reflection of light on the object, and uniformity of lighting [119].

The implementation of algorithms and statistical methods is important to estimate disease severity using image analysis [119]. Algorithms such as support vector machine can be used to reduce the incidence of false positives and false negatives, thus improving detection accuracy [129]. ASSESS is an important image analysis software in the field of plant pathology and it is aimed at measuring plant diseases [129]. This software has filters, contrast and colour saturation functions, as well as colour balancers to enhance the area of interest. It has high measurement accuracy and it also has an automated function [130]. There are several image analysis software that can differentiate between disease symptoms caused by different pathogens [131–132]. The software takes into account several criteria including lesion shape, lesion size, and texture, but this approach has only been successful in some cases [119]. Several statistical methods have been used to explore the quality of image analysis measurement [119]. Regression analysis has been widely used to assess accuracy, precision, reliability, and reproducibility in image analysis techniques [133–134]. The reliability or precision of the method is determined by the coefficient of determination [119]. Lin's concordance correlation coefficient provides an unbiased and quantifiable method to investigate accuracy and precision in the image analysis technique [134–135]. Analysis of variance (ANOVA) and general linear modelling have also been used to investigate sources of error in disease severity estimation [136]. Another method is the coefficient of variation, which provides a good overall index as to the degree of precision and expresses the experimental error as a percentage of the mean [137].

2.3 Guidelines for Pathogenicity Testing in Plants

In this section, we provide guidelines for fungal pathogenicity testing, detailing the steps for inoculum preparation, host preparation, inoculation, and disease assessment. We provide guidelines for pathogenicity testing based on the application of a modified Koch's postulate with Bradford Hill criteria to incorporate the biological gradient criterion (increased dose of inoculum leading to increased effect using suspension of different concentrations), the consistency criterion (similar outcome in different samples based on cross pathogenicity testing), and the plausibility criterion (the causal interpretation must not conflict with the current knowledge) [133].

2.3.1 Preparation of Inoculum

Pure cultures of the isolates are grown on suitable media at a suitable temperature [116]. An alternating 12 h fluorescent light and 12 h dark cycle can be used to induce sporulation [138]. Colonised mycelium plugs are obtained from the periphery of fungal colonies (0.5–1 cm from 14-day colonies) and, when inoculating, the mycelium plug is placed with the mycelium facing the plant tissue [109]. To harvest the conidia, 1–5 mL of sterilized distilled water is placed onto the culture, which is then gently swirled and scraped [116]. The conidial suspension is filtered through two layers of muslin to remove mycelium [116]. Conidial suspension of three different concentrations is prepared and the spore density is adjusted using a haemocytometer (10^4 spores/mL; 1×10^6 spores/mL; 2×10^6 spores/mL). Conidial viability is evaluated by plating three aliquots (around 10 µL) of the suspension on suitable media and checking for further growth [139]. Only conidial suspensions with fungal growth are used for pathogenicity testing [139].

2.3.2 Host Preparation

All the experiments for the attached and detached methods should have replicates as mentioned below. A suitable part of the host is selected based on where the initial disease was observed [116]. It is recommended to use whole plants with at least three replicates (similar age as diseased host) for pathogenicity testing, but where unavailable, a suitable part of the host can be used in the detached method [114, 116].

For the attached method, the suitable parts of the host are cleaned and surface sterilized as described below.

2.3.2.1 Fruit and Leaf Preparation

For the detached method, it is recommended to have at least three replicates for fruits and leaves per treatment [114]. Freshly harvested, untreated, mature but unripe fruits (similar fruits as the initial infection), and leaves (similar size) are washed under running tap water for 1 min [116]. In the laminar flow cabinet, the fruits are surface-sterilized by washing in 70% ethanol for 3 min, then in 1% sodium hypochlorite for 3 min, followed by rinsing with sterilized distilled water three times [116]. The samples are surface dried with sterilized tissue paper and allowed to air-dry on sterilized filter paper [140].

2.3.2.2 Petiole Preparation

For the detached method, petioles are cut to a uniform length (around 4 cm) [141]. The side leaflets are removed, leaving the petiole with only a central leaflet [141]. The petiole with the leaflet is surface-sterilized with 70% ethanol, then in 10% sodium hypochlorite for 10 min and allowed to air dry [103]. It is recommended to have at least 10 petioles per treatment [103].

2.3.2.3 Cane and Stem Preparation

For the detached method, it is recommended to have at least 10 canes or stems per treatment (similar age and diameter as diseased host), which are cut to a uniform length (around 30 cm) [142]. All the leaves, prompt lateral branches, and tendrils are removed. They are surface-sterilized with 70% ethanol, then in 10% sodium hypochlorite for 10 min, and allowed to air dry [142]. This method can also be used for vascular pathogens. In the attached method for vascular pathogens, healthy potted plants with healthy roots are used [142]. The roots are washed to remove soil particles and transferred to pots containing sterilized soil. The shoot region to inoculate the vascular pathogen is surface sterilized with 70% ethanol, then in 10% sodium hypochlorite for 10 min, and allowed to air dry [142].

2.3.2.4 Seed Preparation

The seeds are usually surface-sterilized by submerging in 0.1% Triton X-100 or 0.05% Silwet L-77 for 2 min, then in 0.5% sodium hypochlorite for 2 min, and 70% ethanol for 2 min (depending on the seeds) [143]. The seeds are rinsed three times

in sterile distilled water and dried in sterilized paper towels [143]. Alternatively, the seeds can be surface-sterilized by submerging in 70% ethanol for 3 min, then in 2% sodium hypochlorite for 3 min, followed by rinsing with sterile distilled water three times and being allowed to air dry on sterilized filter papers in a laminar flow cabinet [144]. It is recommended to have at least 16 seeds per treatment [143].

The effectiveness of the surface sterilization method can be evaluated by plating aliquots of the final wash or by lightly pressing individual seeds on media plates and incubating the plates at room temperature for 10 days [145]. The disinfection is considered successful when no fungal growth is observed in the plate and seeds with fungal growth are discarded [145].

2.3.2.5 Root Preparation

Pathogenicity testing for root pathogens depends on the type of the host and the time for the host to develop an adequate root system [109]. In the first method, seeds are surface sterilized as described and sown in sterilized soil [67]. After several weeks, the plants are carefully uprooted and the roots are washed to remove soil particles, surface sterilized with 1% sodium hypochlorite for 1 min, and washed thoroughly with sterile distilled water [146]. In the second method, seedlings (several years old) are acquired and only plants with asymptomatic roots are used for inoculation [109]. It is recommended to have at least five plants per treatment [109].

2.3.3 Inoculation

There are several inoculation methods for leaves and young shoots, which include spraying the spore suspension on the plant tissue [147], dipping the detached plant material into the spore suspension [139], or adding the spore suspension to the detached plant material [148]. The surface-sterilized fruits, leaves, or other parts of the plants are inoculated using the wound and non-wound technique [149].

For the attached method, buds, young shoots, and stems of woody plants can be inoculated by pricking with autoclaved map pins for the wound technique (16 mm needle length with ball-shaped top grip) [78]. A map pin is used to prick a hole in the buds, shoots, or stems and the same pin is used to pick up the inoculum, which is reinserted into the hole [78]. Sterile pins without inoculum are placed into control plants. The pins are left standing throughout the experiment until symptoms develop

(usually 3–12 weeks) [78]. The different tissues can also be wounded using a sterilized blade and inoculated using conidial suspension or mycelium plugs as described below. For the control and non-wound technique, the different tissues are inoculated as described below. The inoculated plants are placed in a ventilated room at 25 °C [78]. All the experiments are repeated at a similar temperature and relative humidity to the collection site.

2.3.3.1 Fruit and Leaf Inoculation

For the wound technique, the middle portion of the fruits and leaves are wounded using a sterilized blade [150]. The wounded fruits and leaves are inoculated by placing 6 µL of conidial suspension of different concentrations (10^4 spores/mL; 1×10^6 spores/mL; 2×10^6 spores/mL) or mycelium plugs (2–5 mm) onto the wound [116]. The control fruits and leaves are inoculated with 6 µL of sterile distilled water or uncolonized plugs [116]. For the non-wound technique, 6 µL of conidial suspension of different concentrations is placed in the middle portion of the samples [138]. The infection site is covered with parafilm to maintain humidity at the start of the experiment, usually for 24–48 h (depending on the pathogen) [138]. All the inoculated samples are incubated individually in a moist chamber at 25 °C with a relative humidity of 80–90% for 7–14 days (or until symptoms develop, depending on the pathogen) [138].

2.3.3.2 Petiole Inoculation

In the detached method, the petioles are placed in centrifuge tubes containing 0.5 mL mycelial conidial suspension of different concentrations [141]. The tubes are placed in a cryogenic storage box, which is then placed in a plastic box containing sterilized wet paper towels [141]. The top of the box is sealed using parafilm and the box is incubated in a greenhouse at 25 °C. This method can also be used to inoculate leaves [141].

2.3.3.3 Cane and Stem Inoculation

Stem inoculation can be carried out by cutting open the bark and placing a conidial suspension of different concentrations or mycelium plug onto the wounded site [141]. The site is covered with parafilm to hold the inoculum [151]. Canes and stems can also be wounded using a 4 mm cork borer or a sterile disposable hypodermic needle [144]. The wounds are inoculated with 1 mL conidial suspension (different

concentrations) or mycelium plugs. The wounds are covered with 100% pure Vaseline petroleum jelly and then wrapped with parafilm [151]. In the non-wounded methods, conidial suspension (different concentrations) or mycelium plugs are used to inoculate the samples, which are wrapped with parafilm [116]. The control canes and stems are inoculated with sterile distilled water or non-colonized plugs [76]. The inoculated canes and stems are then placed in transparent plastic containers containing sterilized wet paper towels to maintain a humid environment [151]. The inoculated samples are incubated at room temperature and inspected for lesion development [151]. For grapevine, the inoculated cuttings can be planted in individual pots containing sterilized soil or they can be placed in tubes containing sterile distilled water and placed in a greenhouse at 25 °C [141, 152]. This detached method can also be used for vascular pathogens, whereby a 4 mm cork borer is used to wound the stem [151]. The wounded stems are inoculated using conidial suspension (different concentrations) or mycelium plugs. In the attached method, a wound is made (using a 4 mm cork borer) on the stem at around 10 cm above the soil level [149]. The wounds are inoculated with mycelium plugs of the vascular pathogen (uncolonized plugs for control) and covered with 100% pure Vaseline petroleum jelly, which are wrapped with parafilm [149].

2.3.3.4 Seed Inoculation

Seed inoculation can be performed using dry conidia, soaking, or soil drenching [153–155]. The surface-sterilized seeds are soaked in conidial suspension of different concentrations containing 0.05% Silwet L-77 and, for the control, the seeds are soaked in sterile water [153]. The seeds are soaked in the dark at 25 °C for 24 h. The surface-sterilized seeds are planted in sterilized germination trays or plastic pots containing sterilized soil and are placed in a greenhouse at 25 °C under natural light to allow germination and growth [153]. The trays are watered when needed. In the second method, the surface-sterilized seeds are coated with the pathogenic strain by shaking them with dry conidia on a shaker at 80 rpm for 10 min before planting them [154]. In the third method, the seeds are first planted in germination trays or plastic pots containing sterilized soil. The soil in each germination tray or plastic pots is inoculated with 100 mL of the conidial suspension (different concentrations) containing 0.05% Silwet L-77, and the control is inoculated with 100 mL of sterile distilled water

containing 0.05% Silwet L-77 [155]. They are then placed in a greenhouse at 25 °C under natural light and watered as needed [155].

2.3.3.5 Root Inoculation

The surface-sterilized roots are inoculated by placing colonised mycelium plugs or conidial suspension of different concentrations on the roots and wrapping them with parafilm [156]. For the wound technique, the roots are wounded using a sterilized blade and inoculated by placing colonised mycelium plugs or conidial suspension (different concentrations) on the wound and wrapping them with parafilm [156]. For the control, sterile distilled water or uncolonized mycelium plugs are used. The inoculated samples are planted in pots containing sterilized soil or placed in tubes containing 40 mL Hoagland solution [156]. The inoculated samples are placed in a greenhouse at 25 °C under natural light and watered as needed [156].

2.3.4 Disease Assessment and Re-Isolation

Disease assessment is usually based on the evaluation of disease symptoms such as the appearance of lesion and the size of lesion according to their severity or incidence [157]. Disease severity is based on the percentage of the relevant host tissue or organ covered by symptoms, whereas disease incidence refers to the percentage of diseased plants or plant parts in the sample irrespective of their severity [158]. However, several factors can affect the estimate of severity including the size and shape of lesions as well as the colour and number of lesions [158]. Another important limitation is that the depth of the lesion is not taken into account in disease assessment [157–158].

We recommend including data from cross pathogenicity testing for all plant disease assessments. The disease reactions of the samples are evaluated from 7 to 14 days (up to several weeks depending on the tissue) after inoculation based on the disease symptoms [76]. The inoculated regions are cut longitudinally through the point of inoculation and the extent of necrosis is measured from the point of inoculation, excluding the wounded region from the measurements [142]. The area of the diseased tissue is calculated based on the length, width, and depth of the diseased symptoms. To evaluate the disease reaction of the root, the plants are carefully uprooted and the roots are washed to remove soil particles, surface sterilized using 2% sodium hypochlorite

for 1 min, and washed thoroughly using sterile distilled water [67]. Root volume serves as a measure of the amount of root tissue lost due to rot [125].

Pathogenicity data are subjected to one-way analysis of variance [152]. It is recommended to assess disease severity using image analysis or visible-wavelength photography as the implementation of both image analysis and visual inspection will provide better accuracy and precision of severity measurement. Disease assessment is evaluated based on measurement of incidence and severity for visual inspection [156].

$$Disease\ Incidence = \left(\frac{Number\ of\ infected\ plant\ units}{Total\ number\ of\ plant\ units\ assessed}\right) \times 100$$

$$Disease\ severity = \left(\frac{Area\ of\ diseased\ tissue}{Total\ tissue\ area}\right) \times 100$$

Aggressiveness is evaluated using the following disease severity scale for canes, fruits, leaves, petioles, seeds, and stems (Table 2.1 modified from [76]). We have modified the category and severity score from Cai et al. [76], which was only applicable to fruits.

Table 2.1 Disease severity scale for canes, fruits, leaves, petioles, seeds, and stems.

Category	Severity
1	no symptoms
2	1–2% of the sample with a necrotic lesion
3	>2 to 5% of the sample with a necrotic lesion
4	>5 to 10% of the sample showing a necrotic lesion
5	>10 to 25% of the sample covered with a necrotic lesion
6	>25 to 50% of the sample showing necrosis
7	>50 to 75% of the sample showing necrosis
8	>75% of the sample showing necrosis

Aggressiveness is evaluated using the following disease severity scale for roots (Table 2.2 modified from [146]). We have modified the category and severity score from Al-Sadi et al. [146], which included nominal scales for the disease severity, which can be subjective [120]. Table 2.2 includes five categories compared with eight categories in Table 2.1, as it includes three categories for disease severity under 10%. This is to allow the application of one scale accurately to individual plant organs (canes, fruits, leaves, petioles, seeds, and stems), which are of different sizes and are structurally different [120]. Only five categories are used to assess disease severity for roots, as most studies have used up to five severity categories and over 50% root with lesion are deemed as severe.

Table 2.2 Disease severity scale for roots

Category	Severity
1	no symptoms
2	1–10% of the root with a necrotic lesion
3	>11 to 25% of the root with a necrotic lesion
4	>25 to 50% of the root with a necrotic lesion
5	>50% of the root with a necrotic lesion

Koch's third postulate is confirmed by re-isolating the inoculated fungus [152]. Pieces of the inoculated tissue are dissected from leading edges for re-isolation [67]. The identity of the re-isolated fungi is confirmed based on the disease symptoms as well as by sequencing the appropriate genes where possible. All inoculated samples are autoclaved twice before disposal [152].

2.4 Concluding Remarks

Fungal plant pathogens can cause serious host damage, but pathogenesis results from a complex interaction between the host immune system and the microorganisms that form part of the microbiota [83]. A polyphasic approach based on morphology,

ecology, and molecular-based approaches is recommended to accurately identify pathogens as the scientific name links the knowledge concerning the species, which is vital to understand the epidemiology and to develop effective quarantine measures [152, 156, 159]. The Bradford Hill criteria and Koch's postulates have several limitations, but they hold major importance in pathogenicity testing, especially for plant pathogens [87, 93]. We provide guidelines for pathogenicity testing based on the application of an updated Koch's postulate with Bradford Hill criteria to include the biological gradient criterion, the consistency criterion, and the plausibility criterion, whereby the causal interpretation must not conflict with the current knowledge [93]. We provide guidelines concerning testing different parts of the plant along with a guide for the severity score, which will lead to a uniform approach to pathogenicity testing and allow inter-studies comparison. The importance of using a suitable sample size to generate accurate mean and reliable results is reinforced [160]. We recommend assessing disease severity using visual inspection and image analysis as the implementation of both methods will provide better accuracy and precision of severity measurement. It is also important to use statistical analyses to investigate sources of error in disease severity estimation from both methods [152]. It is important to note that these are meant to guide causal inference and they should not be used as a heuristic for assessing causation, as there are no set rules that can account for all causations. Several pathogens do not fulfil all the requirements of Koch's postulates as they cannot be cultured or they cannot produce the disease anew, but these pathogens are accepted as the cause of diseases with which they are associated [52]. Therefore, each criterion should be applied and interpreted based on each unique situation. We recommend all studies introducing novel plant pathogenic fungal species to include data on pathogenicity testing and we advise the use of the term *associated with the "disease symptoms" of "the host plant*" in studies that introduce novel species without proving the taxon is the causal agent using Koch's postulates. Where possible, details of disease symptoms should be clearly articulated and a high-resolution image of the symptoms should be included for future reference.

2.5 Future Prospects

Several new methods are being developed to detect and confirm the pathogenic ability of microorganisms, especially those that currently cannot be cultured [119]. Multiplex PCR and real-time PCR have been used to amplify DNA regions coding for specific genes of the targeted pathogen [160]. A major limitation of most PCR techniques is that they cannot differentiate between viable and non-viable cells that led to the development of reverse transcriptase PCR, which is capable of differentiating viable cells [161]. Whole-genome sequencing technologies have also been used to study pathogens [162]. They have provided insights into genes that could be responsible for certain pathogenic traits, but the expression of these genes and the production of a diseased phenotype strongly depends on physiological and ecological factors [163]. Comparative genomics tools have been used in combination with several databases to assign putative functions to products of known or predicted fungal genes [159]. The identification of virulence factors is important to understand the complex processes involved in disease initiation, host immune activation, and the ability of the pathogen to cause an infection [159]. These approaches can be important to target pathogens from uncultured taxa detected by high throughput methods [159]. A major limitation for DNA-based approaches remains the absence of viable microorganism with which to reproduce the disease to satisfy Koch's third postulate, but the insights from these genomic data are vital for the development of diagnostic tools for rapid and effective responses to disease outbreaks [164].

CHAPTER 3

A POLYPHASIC APPROACH TO DELINEATE SPECIES IN *BIPOLARIS*[2]

Abstract

Bipolaris species are important plant pathogens with a worldwide distribution in tropical and temperate environments. Species recognition in *Bipolaris* has been problematic due to a lack of molecular data from ex-type cultures, the use of few gene regions for species resolution and overlapping morphological characters. In this study, we evaluate the efficiency of different DNA barcodes in species delimitation in *Bipolaris* by phylogenetic analyses, Automatic Barcode Gap Discovery and Objective Clustering. GAPDH is determined to be the best single marker for the genus. These approaches are used to clarify the taxonomic placement of all sequences currently named as *Bipolaris* in GenBank based on ITS and GAPDH gene sequence data. In checking various publications, we found that the majority of new host records published in the Plant Disease journal between 2010 and 2019 were based on blast searches of the ITS sequences and up to 82% of those records could be erroneous. Therefore, ITS Blast searches of GenBank to name species is not recommended. Editorial boards of journals and reviewers of new record papers should be aware of this problem. In naming Bipolaris species, whether new or known, it is recommended to perform phylogenetic analyses based on GAPDH using the correct taxon sampling for accurate results and the clade should have reliable statistical support.[1]

[2] This chapter was published as follows:

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I declare that my contribution to this chapter was 95%.

At least two additional species are represented by molecular data in GenBank and we provide an updated taxonomic revision of *Bipolaris.* We accept 45 species in *Bipolaris* and notes are provided for all the species including hosts and geographic distribution.

Keywords: Dothideomycetes, Classification, Integrative Taxonomy, DNA barcoding, Pairwise Differences

3.1 Introduction

Bipolaris was introduced by Shoemaker [165] with *B. maydis* as the type species and belongs to Dothideomycetes, Pleosporales, *Pleosporaceae* [60, 166–170]. *Bipolaris* species are pathogens, saprobes or endophytes of a wide range of hosts [62, 171–173]. *Bipolaris* species are important plant pathogens and are distributed across a broad range of environments and are commonly associated with various diseases, such as leaf spots, foliar blights and root rots [60] mainly in high value field crops in the family *Poaceae* [62, 171, 174]. *Bipolaris* species can cause devastating diseases that have led to starvation of human population in several regions of the world, for example the Bengal famine in India (1943–1944) caused by *Bipolaris oryzae* [56, 175].

Bipolaris species were formerly described in *Helminthosporium*, which were later segregated into several genera including *Bipolaris, Curvularia, Drechslera, Exserohilum, Johnalcornia* and *Porocercospora* [171]. The sexual morph of *Bipolaris* is *Cochliobolus,* which is also the older name, however, conservation of the generic name *Bipolaris* was proposed by Rossman et al. [176] and it is also frequently used by plant pathologists. *Bipolaris* and *Curvularia* are morphologically similar and both genera have sexual morphs in *Cochliobolus* [177], hence, distinguishing these two genera can be problematic [172, 178]. The use of morphological and biological species concepts for the identification of *Bipolaris* species is not always accurate. The use of biological species concept has been problematic due to the lack of sexually produced spores in the genus. Most *Bipolaris* species do not produce the sexual

morph in nature or in culture. The lack of ex-type or authenticated sequences is a drawback in the accurate molecular identification of *Bipolaris* species [179–180], however 27 species currently have an ex-type.

Several species of *Bipolaris* have been widely used in biotechnological applications and genetic manipulation which further emphasises the need for accurate identification [60, 181, 182]. Species delimitation and identification based on morphology alone is difficult due to overlapping morphological characters among species [172, 183]. There are currently 132 species epithets in Index Fungorum [184] under *Bipolaris* with only 43 accepted species [185–187] and 15 species transferred to *Curvularia* [172]. The main objective of the present study is to determine how efficient different DNA barcodes are in species delimitation in *Bipolaris* by using three different methods: Phylogenetic analyses, Automatic Barcode Gap Discovery (ABGD) and Objective Clustering. We also provide clarification of the taxonomic placement of species based on morphology or host association. The study also aims to determine the best marker for the genus and to use the aforementioned methods to delimitate all the species currently named as *Bipolaris* species in GenBank. An overview of the currently accepted species in the genus with their hosts and geographic distribution is also provided. We also aim to provide guidelines on how to delineate species and establish a new taxon in this genus.

3.2 Materials and Methods

3.2.1 Taxon Sampling

This study uses the type species included in Jayawardena et al. [186] and Raza et al. [187] as the starting point. The sequence dataset included the ITS, GAPDH and TEF-1α gene regions of all the 43 species that are currently accepted in *Bipolaris* (Table 3.1).

Table 3.1 Type strains used for the analyses in this study. Ex-type/ex-epitype or reference strains are marked with an asterix (*)

Species	Isolate	GenBank accession numbers		
		ITS	GAPDH	TEF1-α
Bipolaris austrostipae	BRIP 12490*	KX452442	KX452408	KX452459
B. axonopicola	BRIP 11740*	KX452443	KX452409	KX452460
B. bamagaensis	BRIP 13577*	KX452445	KX452411	KX452462
B. bicolor	CBS 690.96	KJ909762	KM042893	KM093776
B. brachiariae	CPC 28819*	MF490806	MF490828	MF490850
B. chloridis	BRIP 10965*	KJ415523	KJ415423	KJ415472
B. clavata	BRIP 12530*	KJ415524	KJ415422	KJ415471
B. coffeana	BRIP 14845*	KJ415525	KJ415421	KJ415470
B. cookei	AR 5185	KJ922391	KM034833	KM093777
B. crotonis	CBS 274.91	KJ909768	KM034820	KM093758
B. cynodontis	CBS 109894	KJ909767	KM034838	KM093782
B. distoseptata	CGMCC3.19361*	MN215628	MN264064	MN263922
B. drechsleri	CBS 136207*	KF500530	KF500533	KM093760
B. gossypina	BRIP 14840*	KJ415528	KJ415418	KJ415467
B. heliconiae	BRIP 17186*	KJ415530	KJ415417	KJ415465
B. heveae	CBS 241.92	KJ909763	KM034843	KM093791
B. luttrellii	BRIP 14643*	AF071350	AF081402	-
B. maydis	CBS 137271*	AF071325	KM034846	KM093794
B. microconidica	CGMCC3.19336*	MN215630	MN264066	MN263924
B. microlaenae	CBS 280.91*	JN601032	JN600974	JN601017
B. microstegii	CBS 132550*	JX089579	JX089575	KM093756
B. oryzae	MFLUCC 10-0715*	JX256416	JX276430	JX266585
B. panici-miliacei	CBS 199.29*	KJ909773	KM042896	KM093788
B. peregianensis	BRIP 12790*	JN601034	JN600977	JN601022
B. pluriseptata	BRIP 14839*	KJ415532	KJ415414	KJ415461
B. sacchari	ICMP 6227	KJ922386	KM034842	KM093785
B. saccharicola	CBS 155.26*	KY905674	KY905686	KY905694
B. salkadehensis	Bi 4*	AB675490	-	-
B. salviniae	BRIP 16571*	KJ415535	KJ415411	KJ415457
B. secalis	BRIP 14453*	KJ415537	KJ415409	KJ415455
B. setariae	CBS 141.31	EF452444	EF513206	-
B. shoemaker	BRIP 15929*	KX452453	KX452419	KX452470
B. simmondsii	BRIP 12030*	KX452454	KX452420	KX452471

Table 3.1 (continued)

Species	Isolate	GenBank accession numbers		
		ITS	GAPDH	TEF1-α
B. sivanesaniana	BRIP 15847*	KX452455	KX452421	KX452472
B. sorokiniana	CBS 110.14	KJ922381	KM034822	KM093763
B. subramanianii	BRIP 16226*	KX452457	KX452423	KX452474
B. urochloae	ATCC 58317	KJ922389	KM230396	KM093770
B. variabilis	CBS 127716*	KY905676	KY905688	KY905696
B. victoriae	CBS 327.64*	KJ909778	KM034811	KM093748
B. woodii	BRIP 12239*	KX452458	KX452424	KX452475
B. yamadae	CBS 202.29*	KJ909779	KM034830	KM093773
B. zeae	BRIP 11512IsoP*	KJ415538	KJ415408	KJ415454
B. zeicola	FIP 532*	KM230398	KM034815	KM093752
Curvularia buchloes	CBS 246.49*	KJ909765	KM061789	KM196588
Curvularia subpapendorfii	CBS 656.74*	KJ909777	KM061791	KM230403

3.2.2 Phylogenetic Analyses and Species Recognition

All analyses conducted in this study were initially performed using a single locus followed by the combined dataset. The sequences were aligned by MAFFT v. 7.036 with the web server (https://mafft.cbrc.jp/alignment/server/) using default settings. They were then edited and improved manually when necessary, using BioEdit v. 7.0.5.2 [188]. Nucleotide statistics and pairwise distances were calculated by the K2P value in MEGA 6.

Phylogenetic reconstructions of concatenated and individual gene trees were performed using maximum Likelihood, maximum Parsimony and Bayesian inference method. The RAxML-HPC2 on XSEDE (8.2.8) [189] in the CIPRES Science Gateway platform [190] was used to perform maximum likelihood analysis by running 1000 pseudoreplicates. Maximum parsimony analysis was conducted using PAUP v.4.0b 10 [191] with the heuristic search option and number of replications 1000 each. The Tree Length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were documented. Kishino-Hasegawa tests (KHT) [192] were performed with aim of determining whether the trees differed significantly. Bayesian inference analysis was conducted

using MrBayes v. 3.1.2 [193]. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The first 20% of generated trees representing the burn-in phase of the analyses were discarded and the remaining 80% of trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree. All resulting trees were viewed with FigTree v.1.4.0 [194] and the final layout was done with Adobe illustrator CS5 (Version 15.0.0, Adobe, San Jose, CA).

The Automatic Barcode Gap Discovery (ABGD) method was used using the online version (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). It was paired with the K2P model, following the default settings, to sort the aligned sequences into hypothetical species, with a preceding P value set at 0.1. ABGD is an analytical tool which detects a barcode gap in the distribution of pairwise genetic distances and determine the number of molecular operational taxonomic units (OTUs) within the dataset [23, 31]. ABGD can also be used when the intra- and inter-specific genetic distances overlap [31, 195]. Once a barcode gap is computed, the dataset is partitioned into groups of sequences, i.e. candidate species. A two-phase system is applied in ABGD, namely, the primary partitioning whereby sequences are initially divided into OTUs based on a barcode gap inferred statistically and secondly, the recursive partitioning, where each sub partition undergoes rounds of splitting until no more split is made and no further significant gaps are found [23, 31, 195].

Objective Clustering in Species Identifier software (TaxonDNA 1.6.2) was next applied to the aligned sequences at a 1-7% threshold to determine the number of OTUs [32]. This method uses pairwise distances to determine the intra- and inter-specific genetic distances of cluster sequences [32–33]. The Species Identifier software is an interface where users can, after having set a threshold, compare the generated clusters with existing taxonomy [33, 196–199].

3.2.3 Species Delineation of all Sequences on GenBank

All ITS and GAPDH sequences currently designated as Bipolaris species in GenBank were downloaded. The sequences were aligned by MAFFT v. 7.036 and improved manually using BioEdit. The ITS and GAPDH datasets were also analysed

using phylogenetic reconstruction using maximum likelihood, the ABGD method and objective clustering method.

3.3 Results

3.3.1 Barcode Analysis

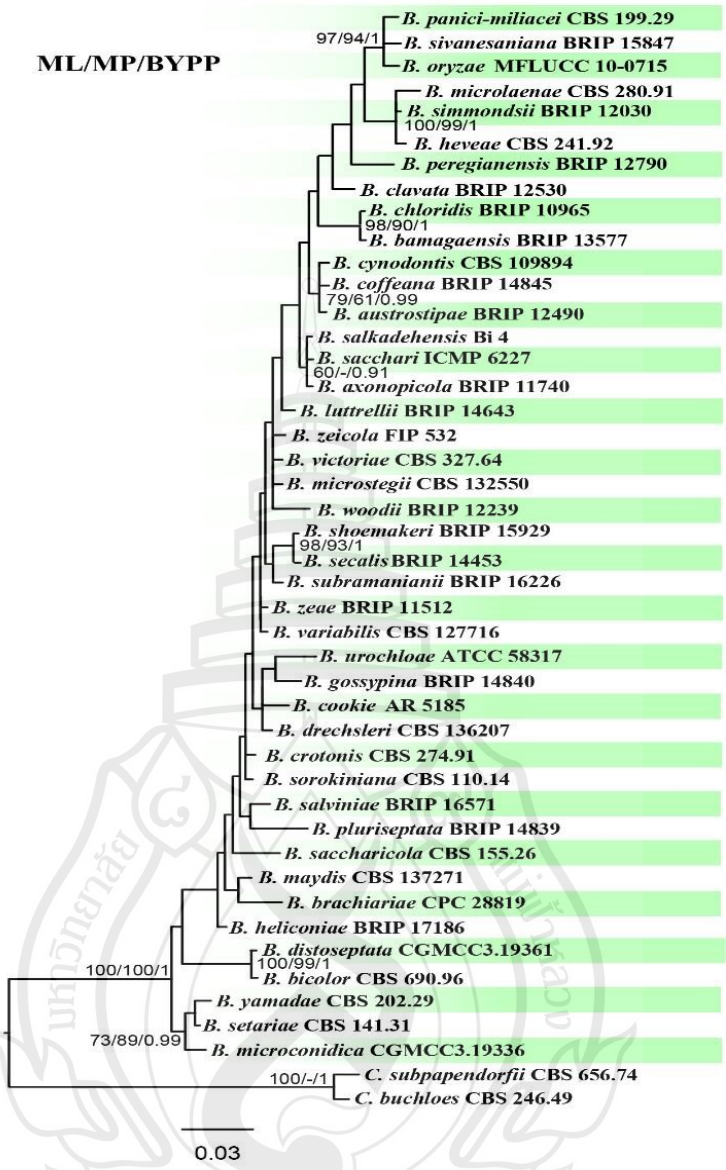
Base composition is an important property of genomes which is related to gene function and regulation. The A-T base pair has two and the G-C base pair has three hydrogen bonds which makes them one of the main parameters to describe DNA base composition. In the ITS dataset, there were 444/581 conserved sites, 128/581 variable sites, of which 84/581 were parsimony informative. The dataset included 43 type sequences of *Bipolaris* and two outgroup taxa (*Curvularia buchloes* (CBS 246.49) and *Curvularia subpapendorfii* (CBS 656.74)). The GAPDH dataset consists of 338/492 conserved sites, 152/492 variable sites, of which 112/492 were parsimony informative. The dataset included 42 type sequences of *Bipolaris* including two outgroup taxa. The GAPDH gene regions are not available for *Bipolaris salkadehensis*. In the TEF-1α dataset, there were 774/877 conserved sites, 102/877 variable sites, of which 58/877 were parsimony informative. The dataset included 40 type sequences of *Bipolaris* including two outgroups. The TEF-1α gene regions are not available for *Bipolaris luttrellii, B. salkadehensis* and *B. setariae.* The combined dataset (ITS, GAPDH and TEF-1α) consists of 1556/1958 conserved sites, 382/1958 variable sites, of which 254/1958 were parsimony informative. The highest percentage of parsimony informative sites and variable sites occurred in the GAPDH gene region, followed by ITS, the combined dataset and finally the TEF-1α gene region. The ITS region was the most AT-biased compared to other gene regions with an average of 54.3% and the GAPDH gene region is the most informative at 22.76% based on analysis of the nucleotide composition. The highest GC content was observed in the TEF-1α gene region with an average of 58.3% which suggest that it is highly stable.

3.3.2 Species Discrimination

The ITS alignment including the outgroups consisted of 1007 characters which were edited to remove ambiguous region and gaps, resulting in an ITS alignment of 581 characters. The parsimony analysis of the data matrix yielded one most parsimonious tree out of 176 (CI = 0.639, RI = 0.780, RC = 0.498, HI = 0.361, Tree Length = 249). The maximum parsimony and Bayesian inference trees were similar to the maximum likelihood tree (Figure 3.1) in terms of major clades. However, the phylogenetic position of some species varied between the different methods but these are not discussed further as the nodes supporting those species were not well supported.

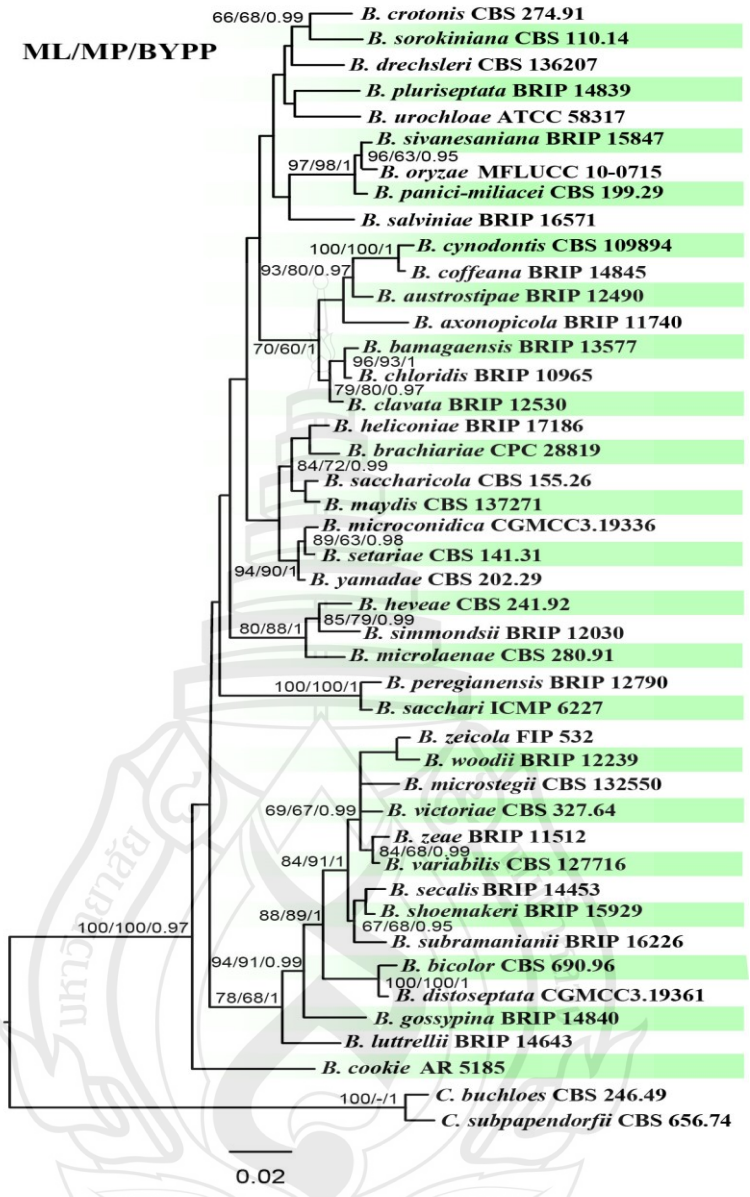
The GAPDH alignment including the outgroup initially consisted of 608 characters which was edited to remove ambiguous region and gaps, resulting in a final alignment of 492 characters. The parsimony analysis of the data matrix yielded one most parsimonious tree out of 31 (CI = 0.631, RI = 0.773, RC = 0.488, HI = 0.369, Tree Length = 290). The maximum parsimony, Bayesian inference and maximum likelihood analysis (Figure 3.2) resulted in clades with similar topology.

The TEF-1α alignment including the outgroup consisted of 986 characters which was edited to remove ambiguous region and gaps, resulting in a TEF-1α alignment of 877 characters. The parsimony analysis of the data matrix yielded one most parsimonious tree out of 97 (CI = 0.665, RI = 0.724, RC = 0.481, HI = 0.335, Tree length = 164). The maximum parsimony and Bayesian inference trees were similar to the maximum likelihood tree (Figure 3.3) in terms of major clades. However, the phylogenetic position of some species varied between the different methods but these are not discussed further as the nodes supporting those species were not well supported.



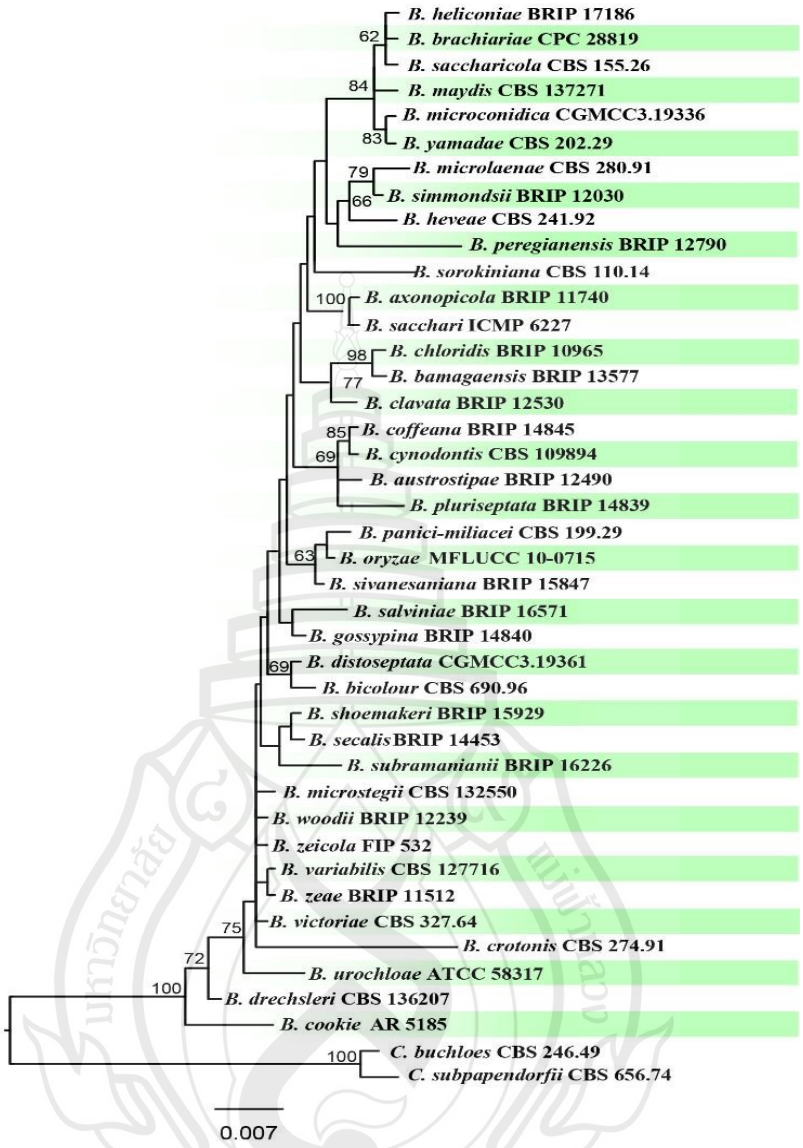
Note Phylogenetic tree generated by maximum likelihood analysis of ITS region of *Bipolaris* type sequences. RAxML bootstrap support and maximum parsimony values ≥ 60% (BT) as well as Bayesian posterior probabilities ≥0.90 (PP) are shown respectively near the nodes. The scale bar indicates 0.03 changes per site and the tree is rooted with *C. buchloes* and *C. subpapendorfii.*

Figure 3.1 Phylogenetic tree of *Bipolaris* based on ITS region



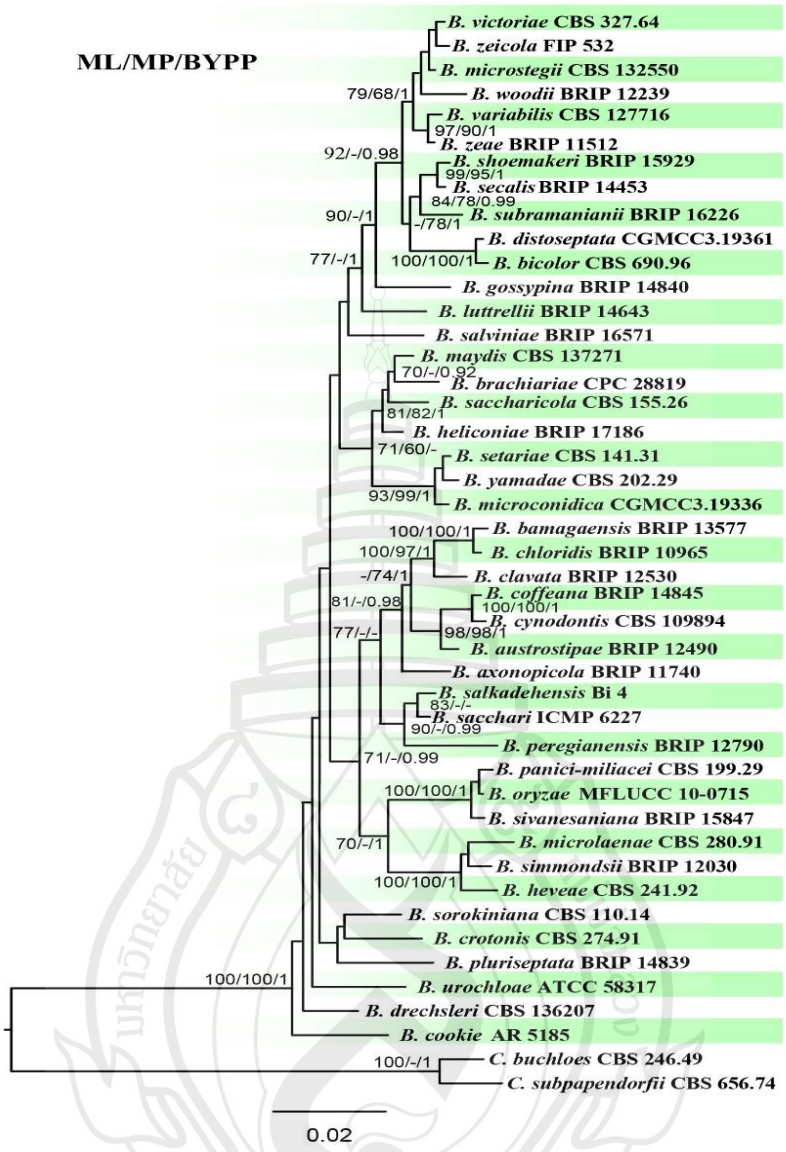
Note Phylogenetic tree generated by maximum likelihood analysis of GAPDH gene region of *Bipolaris* type sequences. RAxML bootstrap support and maximum parsimony values ≥ 60% (BT) as well as Bayesian posterior probabilities ≥0.90 (PP) are shown respectively near the nodes. The scale bar indicates 0.02 changes per site and the tree is rooted with *C. buchloes* and *C. subpapendorfii.*

Figure 3.2 Phylogenetic tree of *Bipolaris* based on GAPDH gene



Note Phylogenetic tree generated by maximum likelihood analysis of *TEF1-α* sequence data of *Bipolaris* type sequences. RAxML bootstrap support and maximum parsimony values ≥ 60% (BT) as well as Bayesian posterior probabilities ≥0.90 (PP) are shown respectively near the nodes. The scale bar indicates 0.007 changes per site and the tree is rooted with *C. buchloes* and *C. subpapendorfii.*

Figure 3.3 Phylogenetic tree of *Bipolaris* based on *TEF1-α* gene



Note Phylogenetic tree generated by maximum likelihood analysis of combined ITS, GAPDH and TEF1-α sequence data of *Bipolaris* type sequences RAxML bootstrap support and maximum parsimony values ≥ 60% (BT) as well as Bayesian posterior probabilities ≥0.90 (PP) are shown respectively near the nodes. The scale bar indicates 0.02 changes per site and the tree is rooted with *C. buchloes* and *C. subpapendorfii.*

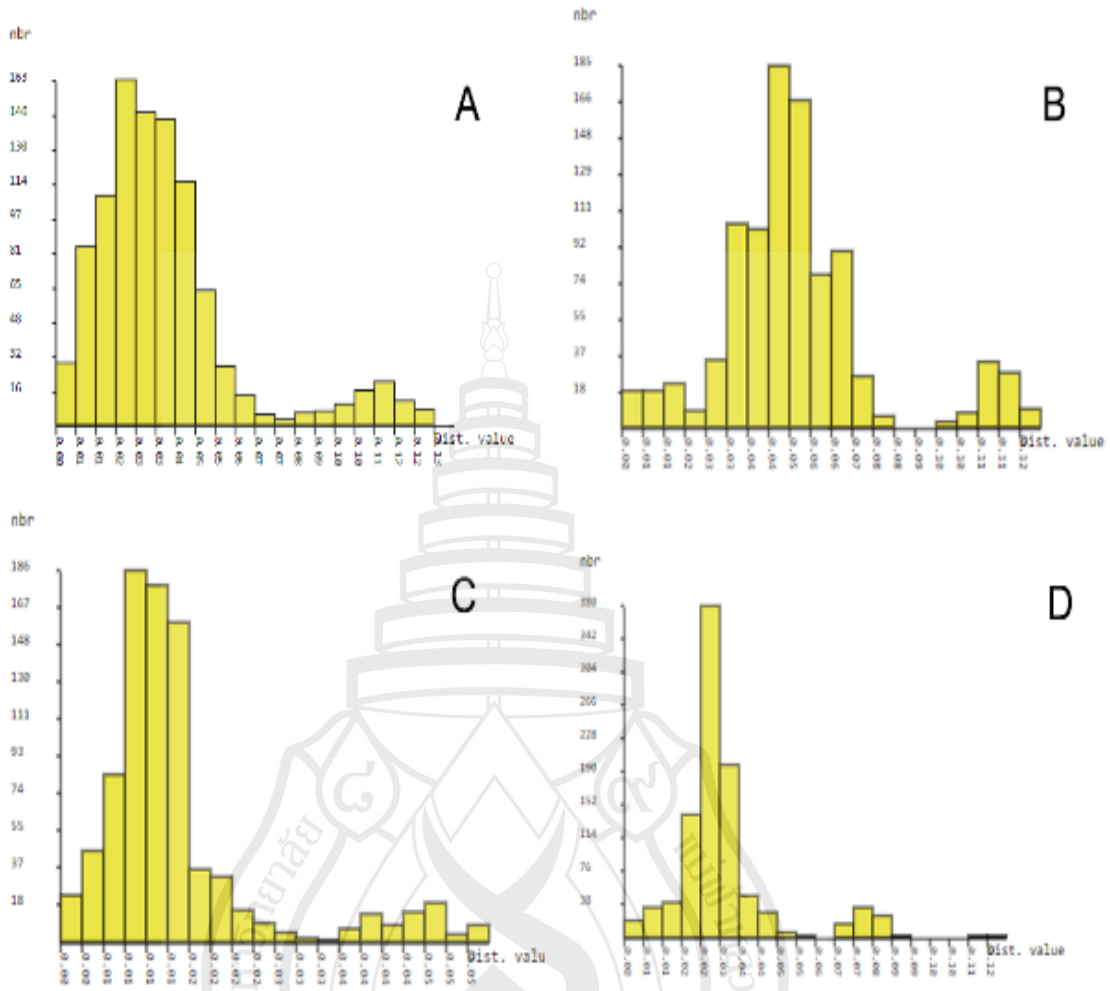
Figure 3.4 Phylogenetic analysis of *Bipolaris* based on combined dataset

The combined dataset (ITS, GAPDH and TEF-1α) consisted of 1958 characters including the outgroup. The parsimony analysis of the data matrix yielded one most parsimonious tree out of 532 (CI = 0.593, RI = 0.715, RC = 0.424, HI = 0.407, Tree Length = 757). The maximum parsimony and Bayesian inference trees were similar to the maximum likelihood tree (Figure 3.4) in terms of major clades and topology of the tree.

All different phylogenetic approaches resulted in similar tree topology and major clades, although the position of some of the species varied between the different methods for the ITS and TEF-1α dataset. Phylogenetic analysis based on the GAPDH dataset resulted in clades with similar topology based on all three phylogenetic approaches. The topological structure of the ITS and GAPDH tree is similar in terms of major clades to the combined dataset. In terms of single-locus dataset, the topology of the GAPDH tree was most similar to the combined dataset, followed by the ITS tree.

3.3.3 ABGD Species Delimitation

ABGD generated a histogram of the pairwise differences in the GAPDH and the combined dataset (Figure 3.5). The barcode gap represents the difference that can be observed in the distribution between intraspecific diversity and interspecific diversity based on pairwise difference in a dataset. Barcoding gap was observed at K2P distance of 0.08–0.10 in the GAPDH dataset and rough barcode gaps are observed at around 0.06 and 0.09–0.11 in the combined dataset. As no barcoding gap was observed in the ITS and TEF-1α dataset, ABGD cannot propose a primary partition and is therefore not able to correctly delineate species in these datasets and these gene regions are not further analysed using ABGD.

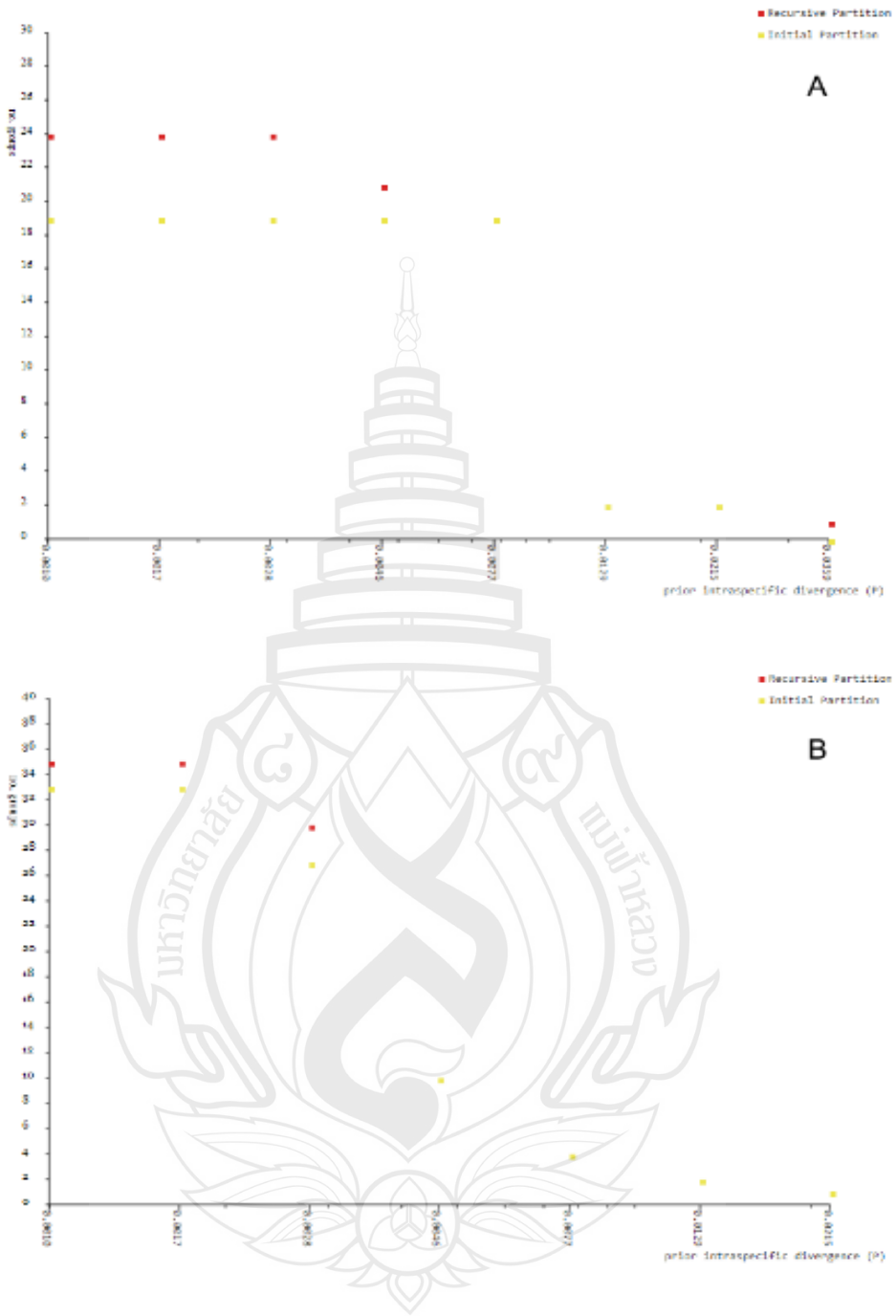


Note Histogram of pairwise distance of the ITS (A), GAPDH (B), TEF-1α (C) and the combined dataset (D). The horizontal axis shows the pairwise K2P-distance and the vertical axis shows the number of pairwise sequence comparisons.

Figure 3.5 ABGD results based on different gene regions

Once a barcode gap is computed, the dataset is partitioned into candidate species. ABGD produced 8 partitions and each partition proposed 2 - 19 putative species (24 when using recursion) in the GAPDH dataset while prior intraspecific divergences (P) varied from 0.001 to 0.0359 (Figure 3.5). As primary partitions are stable on a wide range of prior values and the primary partition value is usually considered close to the hypothetical number of species, the value of 19 was selected as the result of ABGD for the GAPDH dataset. It corresponds to a barcoding gap around 0.08–0.10 (Figure 3.5B) with 19 taxonomic units (Figure 3.6A) which are detailed as follows: *B. austrostipae, B. bamagaensis, B. chloridis, B. clavata, B. coffeana, B. cynodontis* in one group, *B. bicolor, B. distoseptata* in one group*, B. brachiariae, B. heliconiae, B. maydis, B. microconidica, B. saccharicola, B. setariae, B. yamadae* in one group, *B. heveae, B. simmondsii* in one group*, B. microstegii, B. secalis, B. shoemakeri, B. subramanianii, B. variabilis, B. victoriae, B. woodii, B. zeae, B. zeicola* in one group*, B. oryzae, B. panici-miliacei, B. sivanesaniana* in one group*, B. peregianensis, B. sacchari* in one group*, C. buchloes, C. subpapendorfii* in one group.

For the combined dataset, ABGD produced 7 partitions while P varied from 0.001 to 0.0215. In each partition, 2 to 33 putative species (35 when applying recursion) were found. Barcoding gaps in the combined dataset (0.06–0.07, 0.09–0.11) (Figure 3.5D) did not correspond to any taxonomic units (Figure 3.6B). In the partition with the highest number of clusters, *B. axonopicola, B. sacchari, B. salkadehensis* were clustered in one group, *B. bamagaensis, B. chloridis* in one group, *B. bicolor, B. distoseptata* in one group, *B. coffeana, B. cynodontis in one group, B. oryzae, B. panici-miliacei, B. sivanesaniana* in one group, *B. secalis, B. shoemakeri* in one group, *B. variabilis, B. zeae* in one group and *B. victoriae, B. zeicola* in one group. The remaining species were separated in individual groups with each outgroup in one group giving a total of 35 clusters.



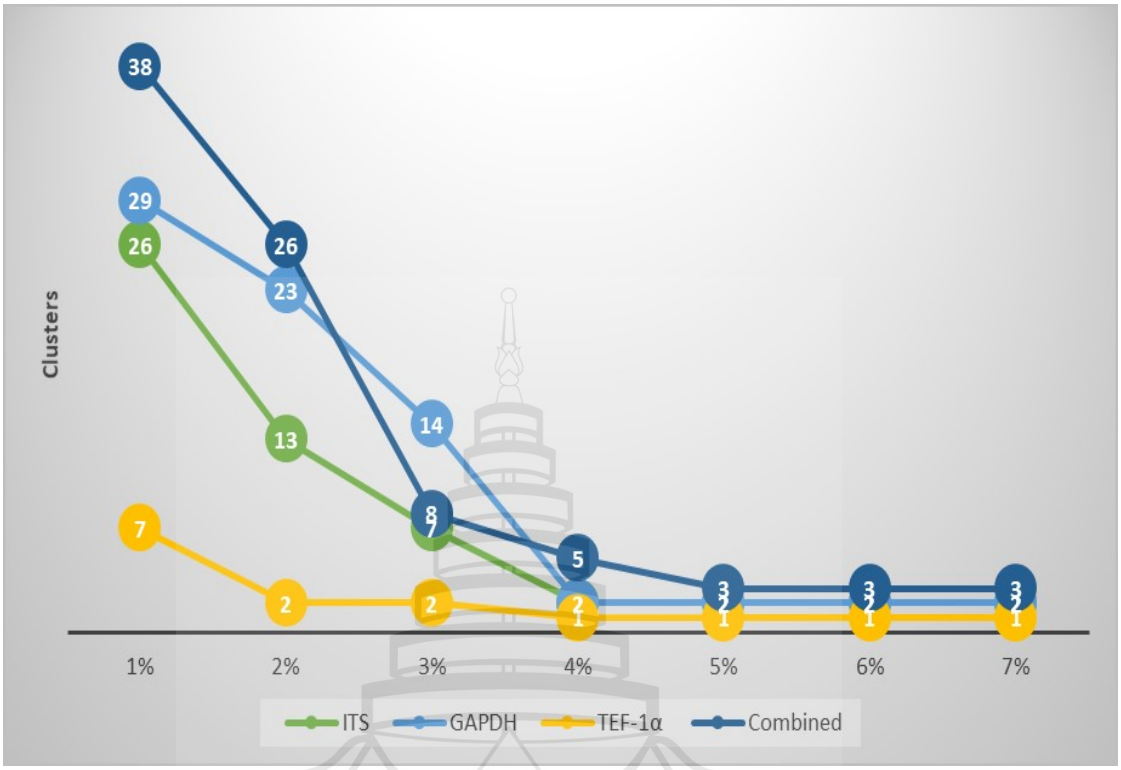
Note Partitions under different prior intraspecific divergences for the GAPDH (A) and the combined dataset (B).

Figure 3.6 ABGD partitions for different datasets

3.3.4 Objective Clustering

The number of clusters in each dataset was estimated using objective clustering (Figure 3.7). The ITS dataset yielded 26 clusters at 1%, 13 clusters at 2%, 7 clusters at 3% and 2 clusters ranging from 4% to 7% threshold. GAPDH yielded 29 clusters at 1%, 23 clusters at 2%, 14 clusters at 3% and 2 clusters ranging from 4% to 7% threshold. TEF-1α yielded 7 clusters at 1%, 2 clusters at 2% to 3% and 1 cluster from 4% to 7% threshold. The combined dataset yielded 38 clusters at 1%, 26 clusters at 2%, 8 clusters at 3%, 5 clusters at 4%, 3 clusters at 5% to 7% threshold. In all the dataset, the outgroups occupied separate clusters to the *Bipolaris* species except for the TEF-1α dataset at 4% to 7% threshold. Objective clustering resulted in the highest number of clusters at 1% threshold with ITS at 26 clusters, GAPDH at 29 clusters, TEF-1α at 7 clusters and 38 clusters for the combined dataset. The percentage threshold at 1% for example means that if a query differs by 1% from another sequence, the query may still belong to the same 26 species in the ITS dataset. Applying the ABGD method with prior intraspecific divergence ranging from 1% – 7% yielded 2 OTUs in the GAPDH and combined dataset, which differs significantly from values of objective clustering.

The ABGD method delineated a relatively low number of species in the GAPDH dataset at 43.18% compared to phylogenetic analyses. Based on the highest number of clusters identified by objective clustering, species delineation was 84.4% in the combined dataset, 65.91% for GAPDH, 57.77% for ITS and 15.56% for TEF-1α. These results are considerably higher compared to the ABGD approach based on our dataset. Objective clustering is in agreement with phylogenetic analysis for TEF-1α as a poor marker for *Bipolaris* as TEF-1α clustered the dataset into two groups, one for *Bipolaris* species and the second one for the outgroups at percentage threshold of 2% - 3%.



Note The number of DNA clusters according to Objective Clustering at different thresholds for ITS, GAPDH, TEF-1α and combined dataset.

Figure 3.7 Objective Clustering for different datasets

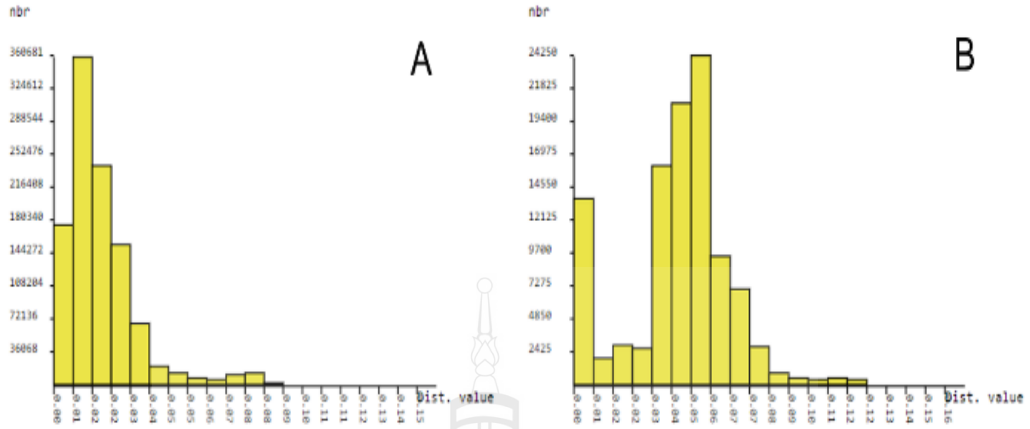
3.3.5 Delineating all Sequences in GenBank

Based on analysis of the type sequences of *Bipolaris*, the two best performing genes (ITS & GAPDH) were used for species delineation for all sequences in GenBank that are currently deposited as *Bipolaris* species. All ITS sequences for *Bipolaris* were downloaded from GenBank (accessed 28th August 2019) and the original dataset included 2098 sequences including two outgroup taxa. All sequences were checked in Index Fungorum [184] (www.indexfungorum.org; accessed 15 September 2019) and 265 sequences were excluded for further analysis as they are not currently classified under *Bipolaris*. Fifty duplicate sequences were deleted from the dataset. Twenty-five sequences were deleted based on the alignment and 38 sequences with long branches in the maximum likelihood tree were deleted. Two-hundred and

forty-eight sequences clustered with the outgroup and were deleted, resulting in a maximum likelihood tree with a final alignment of 1472 sequences including the two outgroup taxa. The sequences were named as per their current annotation on GenBank and based on the phylogenetic analyses. Two hundred and one sequences did not cluster with their respective type sequences, however only 66 sequences had statistical support (≥ 60% BT). The maximum likelihood tree was not informative enough to clearly delineate between some of the type species based on the ITS sequence data thus clustering them together such as between *B. oryzae/B. panici-miliacei, B. austrostipae/B. cynodontis, B. crotonis/B. sorokiniana, B. variabilis/B. zeae* and *B. bicolor/B. distoseptata*. The maximum likelihood tree also showed 57 sequences which formed separate clades to any type species of *Bipolaris* with strong bootstrap support (≥ 60% BT).

All GAPDH sequences for *Bipolaris* were downloaded from GenBank (accessed 4th December 2019) and the original dataset included 607 sequences including two outgroup taxa. All sequences were checked in Index Fungorum [184] and three sequences were excluded from further analysis as they are not currently classified under *Bipolaris*. Forty-one sequences were deleted based on the alignment and six sequences with long branches in the maximum likelihood tree were deleted. Ninety-eight sequences clustered with the outgroup and were deleted, resulting in a maximum likelihood tree with an alignment of 459 sequences including two outgroup taxa. The GAPDH tree resulted in all the type sequences in different cluster, showing good resolution at the species level and three sequences formed separate clades to any type species of *Bipolaris.*

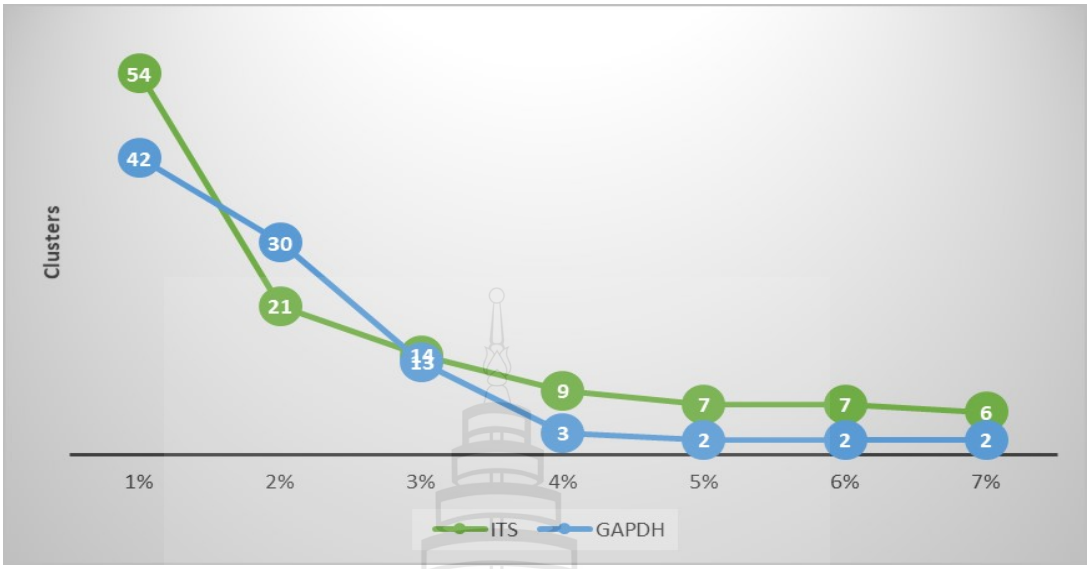
In the ITS dataset, 29.83% sequences downloaded were excluded from analysis as it included sequences that are not currently classified as *Bipolaris*, or duplicate sequences, or sequences that clustered with the outgroup and based on our phylogenetic analysis. Of the ITS sequences. 13.65% did not cluster with their respective type sequences and 3.87% formed separate clades to any accepted type species with moderate support (≥ 60% BT).



Note Histogram of pairwise distance of all the ITS (A) and GAPDH (B) sequences from GenBank. The horizontal axis shows the pairwise K2P-distance and the vertical axis shows the number of pairwise sequence comparisons.

Figure 3.8 ABGD results based on sequences on GenBank

In the maximum likelihood tree based on the ITS type sequences, a number of sequences clustered together forming a clade of closely related species such as *B. oryzae, B. sivanesaniana, B. panici-miliacei*; *B. cynodontis, B. coffeana, B. austrostipae*; *B. salkadehensis, B. axonopicola, B. sacchari; B. sorokiniana, B. crotonis*, and species that formed close relationship to those type species could not be well-differentiated based on the ITS sequence alone. Of the GAPDH dataset, 24.38% of sequences downloaded were excluded from the analysis as it included sequences that are not currently classified as *Bipolaris*, duplicate sequences, sequences that clustered with the outgroup and based on phylogenetic analysis. Of the GAPDH dataset, 9.8% sequences did not cluster with their respective type sequences.



Note The number of DNA clusters according to Objective Clustering at different thresholds for all the ITS and GAPDH sequences of *Bipolaris* on GenBank.

Figure 3.9 Objective Clustering based on sequences on GenBank

The dataset of all ITS and GAPDH sequences from GenBank used for phylogenetic analysis were then analysed using the ABGD method and objective clustering. No gap was observed in any of the two datasets (Figure 3.8), therefore, ABGD may not be able to correctly delineate species in these datasets and they were not further analysed using ABGD.

3.3.6 Cluster Estimation for all *Bipolaris* Sequences in GenBank

The number of clusters in the dataset of all the sequences from GenBank were estimated using objective clustering (Figure 3.9). The ITS dataset yielded 54 clusters at 1%, 21 clusters at 2 %, 14 clusters at 3%, 9 clusters at 4%, 7 clusters at 5% & 6% and 6 clusters at 7 % threshold. GAPDH yielded 42 clusters at 1%, 30 clusters at 2%, 13 clusters at 3%, 3 clusters at 4% and 2 clusters ranging from 5% to 7% threshold. Using the largest dataset resulted in a higher number of clusters being detected in both datasets compared to the type sequences.

In our study, entries that did not cluster with their respective types (≥ 60% BT) were checked if they are associated with refereed journals. We found that the entries are related to a number of publications. In checking various publications, we found that a number of *Bipolaris* entries (53 in total) were published in the Plant Disease journal (Table 3.2) with most entries named based on BLAST searches in NCBI GenBank. Sequences from Gasparetto et al. [185] and Wang et al. [200] were not included in our analysis as the sequences were not available on GenBank when the datasets were downloaded. Three *Bipolaris zeae* strains were named based on phylogenetic analyses of a combined dataset (ITS & GAPDH) of eight *Bipolaris* species [201]. Our analysis using the ITS region suggest the isolates to be *B. variabilis/zeae,* however, the GAPDH gene region placed the isolates (KU571467 & KU571469) as *B. variabilis* with moderate support (ML 64% BT) and confirmed the isolate (KU571468) as *B. zeae* (ML 69% BT). Twenty-three entries had only the ITS region and our phylogenetic analysis confirmed the placement of four entries (MK002698, MF770710, KF150215, & JQ316121) and could not confirm five entries of *B. sorokiniana* and *B. oryzae* (MH592543 clustered with *B. crotonis/B. sorokiniana*; JQ237248, GU222690, GU222691, GU222692 & GU222693 as *B panici-miliacei/B. oryzae*). Of the remaining 12 reports, eight entries (KF041822, GU290228, HQ611957, KJ476182, KJ476183, KJ476184, KC592365 & JF506092) did not cluster with any type sequences, forming separate clades and two entries clustered with *Curvularia* (HQ015445 & KT210885). *Bipolaris* sp. (GU046562) clustered with the type of *B. microstegrii* and *B. setariae* (KJ605157) clustered with *B. yamadai.* Twenty-nine entries had ITS and GAPDH gene regions, however four *B. cactivora* species [199, 202] could not be confirmed based on the ITS and GAPDH gene regions. *Bipolaris spicifera* (ITS/GAPDH: KC897667/KC928089) did not cluster with the respective type, but instead clustered with the outgroup and were deleted from the dataset. *Bipolaris maydis* (ITS/GAPDH: KY426814/KY434196) gene regions clustered with the type of *B. heliconiae* in the current study. *Bipolaris panici-miliacei* (ITS/GAPDH: MF083601/MF112228) placement could not be confirmed based on the ITS region and it clustered with the type of *B. oryzae* in the maximum likelihood tree based on GAPDH gene with moderate support (70% BT).

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Cochliobolus spicifer genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA	926	926	100%	0.0	100.00%	AB678224.1
Curvularia spicifera isolate BCsIAGS internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	891	891	96%	0.0	100.00%	KU379555.1
Curvularia sp. isolate SCUA-Ahw-Vig-1Ra internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed	889	889	96%	0.0	100.00%	MG971269.1
Fungal endophyte isolate SNP013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer	889	889	96%	0.0	100.00%	KP335219.1
Curvularia australiensis strain VKR 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	889	889	96%	0.0	100.00%	KM999998.1
Uncultured Basidiomycota clone Mesq_C07 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA g	889	889	96%	0.0	100.00%	EU490129.1
Uncultured Ascomycota clone Mesq_H03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	889	889	96%	0.0	100.00%	EU490151.1
Uncultured Ascomycota clone Mesq_F12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	889	889	96%	0.0	100.00%	EU490146.1
Uncultured Ascomycota clone C31_H02 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	889	889	96%	0.0	100.00%	EU490088.1
Uncultured Ascomycota clone 4S1_C01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	889	889	96%	0.0	100.00%	EU489973.1
Uncultured Ascomycota clone 4M4_D08 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	889	889	96%	0.0	100.00%	EU489952.1
Uncultured Ascomycota clone 4M1_H09 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	889	889	96%	0.0	100.00%	EU489941.1
Uncultured Ascomycota clone 4M1_E03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	889	889	96%	0.0	100.00%	EU489913.1
Uncultured Ascomycota clone 4M1_B07 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	889	889	96%	0.0	100.00%	EU489892.1
Uncultured zygomycete clone Mesq_E05 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	889	889	96%	0.0	100.00%	EU490136.1
Uncultured zygomycete clone Mesq_C01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	889	889	96%	0.0	100.00%	EU490126.1
Bipolaris maydis 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	889	889	96%	0.0	100.00%	KJ913699.1
Cochliobolus australiensis strain JL-37 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	889	889	96%	0.0	100.00%	JX867233.1
Dothideomycetes sp. genotype 150 isolate AK1298 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal tra	889	889	96%	0.0	100.00%	JQ759635.1
Bipolaris spicifera strain NRRL 47508 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	889	889	96%	0.0	100.00%	GU183125.1

Note Blast search of the sequence HQ015445 with only the first 20 hits being shown.

Figure 3.10 Blast search result

The identification of *Bipolaris* species based on blast search alone have resulted in a number of sequences which could not be confirmed based on phylogenetic analyses. For example, Vu et al. [203] identified their isolate (ITS: HQ015445) as *B. spicifera* based on blast search result showing 100% homology to *B. spicifera* strain NRRL 47508 (GU183125.1). A blast search of the sequence HQ015445 is shown in Figure 3.10 which shows a 100% identity to a number of species including *Curvularia* and in the maximum likelihood tree, HQ015445 clustered with *Curvularia* and was excluded. Another example includes *B. spicifera* isolate [204; ITS/GAPDH: KC897667/KC928089] which was identified based on blast search result showing 99.8% identity to *B. spicifera* strain CCTU 245 (ITS/GAPDH: JX070077 and JX070078).

Table 3.2 List of *Bipolaris* strain sequences deposited in GenBank published as new records in the Plant Disease journal. The table lists the accession number for ITS and GAPDH gene regions and associated reference. The TEF-1α gene regions were not included in the table

Published species name	Correct species name	GenBank details		
		ITS	GAPDH	Reference
Bipolaris cactivora	Unconfirmed	MH725590	-	Garibaldi et al. [202]
B. cactivora	Unconfirmed	KF041822	-	Garibaldi et al. [202]
B. cactivora	Unconfirmed	HM598677	HM598680	Tarnowski et al. [199]
B. cactivora	Unconfirmed	HM598678	HM598681	Tarnowski et al. [199]
B. cactivora	Unconfirmed	HM598679	HM598682	Tarnowski et al. [199]
B. drechsleri	*B. drechsleri*	KU298311	KU298310	Chamorro et al. [205]
B. maydis	*B. heliconiae*	KY426814	KY434196	Huang et al. [206]
B. maydis	Unconfirmed	KJ476182	-	Macedo et al. [207]
B. maydis	Unconfirmed	KJ476183	-	Macedo et al. [207]
B. maydis	Unconfirmed	KJ476184	-	Macedo et al. [207]
B. micropus	Not in GenBank	KY20355	-	Gasparetto et al. [185]
B. microstegii	*B. microstegii*	KF150215	-	Bruckart et al. [208]
B. oryzae	*B. oryzae*	MF185132	MF431722	Huang et al. [209]
B. oryzae	Not in GenBank	MH412646	MH412650	Wang et al. [200]
B. oryzae	*B. oryzae*	MH920540	MH921828	Kaspary et al. [210]
B. oryzae	*B. oryzae*	MG448606	MG458233	Kaspary et al. [210]
B. oryzae	*B. oryzae*	KR738745	MF033264	Li et al. [103]
B. oryzae	*B. oryzae*	KX179475	KX344512	Sanahuja and Palmateer [211]
B. oryzae	*B. oryzae*	KX179476	KX344513	Sanahuja and Palmateer [211]
B. oryzae	*B. oryzae*	KX594827	KX594829	Sanahuja et al. [212]
B. oryzae	*B. oryzae*	KX594828	KX594830	Sanahuja et al. [212]
B. oryzae	*B. oryzae/B panici-miliacei*	JQ237248	-	Vu et al. [203]
B. oryzae	*B. oryzae*	JF693908	JF521648	Waxman and Bergstrom [213]
B. oryzae	*B. oryzae*	JF693909	JF521649	Waxman and Bergstrom [213]
B. oryzae	*B. oryzae*	JF693910	JF521650	Waxman and Bergstrom [213]
B. oryzae	*B. oryzae/B panici-miliacei*	GU222690	-	Tomaso-Peterson and Balbalian [214]

Table 3.2 (continued)

Published species name	Correct species name	GenBank details		
		ITS	GAPDH	Reference
B. oryzae	*B. oryzae/B panici-miliacei*	GU222691	-	Tomaso-Peterson and Balbalian [214]
B. oryzae	*B. oryzae/B panici-miliacei*	GU222692	-	Tomaso-Peterson and Balbalian [214]
B. oryzae	*B. oryzae/B panici-miliacei*	GU222693	-	Tomaso-Peterson and Balbalian [214]
B. panici-miliacei	*B. oryzae*	MF083601	MF112228	Ding et al. [215]
B. papendorfii	Unconfirmed	KC592365	-	Li et al. [103]
B. peregianensis	*B. peregianensis*	JQ316121	-	Wang et al. [216]
B. secalis	*B. secalis*	MF770710	-	Bernardi et al. [217]
B. setariae	*B. setariae*	MK002698	-	Xiao et al. [218]
B. setariae	*B. yamadai*	KJ605157	-	Niu et al. [219]
B. setariae	*B. setariae*	KT805922	KT982612	Liu et al. [220]
B. setariae	Unconfirmed	GU290228	-	Shi et al. [221]
B. sorokiniana	*B. sorokiniana/ B. crotonis*	MH592543	-	Li et al. [222]
B. sorokiniana	*B. sorokiniana*	MK905507	MK929554	Li et al. [222]
B. sorokiniana	*B. sorokiniana*	MH538292	MH538293	Li et al. [222]
B. sorokiniana	*B. sorokiniana*	KU870641	KU870644	Wang and Wei [223]
B. sorokiniana	Unconfirmed	HQ611957	-	Vu et al. [203]
B. sp.	*B. microstegii*	GU046562	-	Kleczewski and Flory [224]
B. sp.	Unconfirmed	JF506092	-	Zhang et al. [225]
B. spicifera	*Curvularia* sp.	KC897667	KC928089	Amaradasa and Amundsen [204]
B. spicifera	*Curvularia* sp.	KT210885	-	Li et al. [222]
B. spicifera	*Curvularia* sp.	HQ015445	-	Vu et al. [203]
B. victoriae	*B. victoriae*	KX371072	MF155552	Tian and Smith [226]
B. zeae	*B. zeae*	KU356179	MF415650	Zhao et al. [227]
B. zeae	*B. zeae*	KU356180	MF415651	Zhao et al. [227]
B. zeae	*B. variabilis*	KU571464	KU571467	Xue et al. [201]
B. zeae	*B. zeae*	KU571465	KU571468	Xue et al. [201]
B. zeae	*B. variabilis*	KU571466	KU571469	Xue et al. [201]

However, based on our phylogenetic analysis, sequences KC897667, KC928089, JX070077 and JX070078 clustered with the outgroup and were excluded from the dataset. The strains listed in Table 3.2 were also subjected to blast search that include sequences from type material only. Of the 23 entries with only the ITS region, only four entries were correctly identified (KF150215, KJ605157 and JQ316121) based on the result with the highest query cover and percentage identity. Of the 29 entries with ITS and GAPDH gene regions, only three entries were correctly identified (GAPDH: MF415651, KU571469 and KU298310) based on the result with the highest query cover and percentage identity. Our study also highlighted issues where blast searches resulted in wrongly identified species, thus resulting in wrong placement at species or genus level.

3.4 Discussion

Our study confirms that the best single marker for *Bipolaris* is GAPDH based on phylogeny, ABGD and objective clustering. This is also in accordance with previous studies that were based only on phylogeny [172, 174]. The single marker GAPDH can therefore be used to delimitate *Bipolaris* species. Our study based on the type sequences suggested that objective clustering performed better as compared to ABGD for species delineation in this genus and both methods gave the best results based on the combined dataset. At a 1% threshold, objective clustering resulted in 29 and 26 clusters for type sequences of GAPDH and ITS regions respectively. However, the number of clusters changed drastically when using all the sequences of *Bipolaris* from GenBank, resulting in 54 clusters and 42 clusters in ITS and GAPDH dataset respectively. These results suggest that the dataset in GenBank consists of a number of new species that have not been correctly identified, which would potentially increase the number of accepted species in this genus if the entries are supported by morphological data.

Data published in the Plant Disease journal based on a blast search of the ITS sequences resulted in 17.39% of those entries being confirmed as correctly named based on our study (4 out of 23 entries). Of the entries with ITS and GAPDH gene

regions (20 out of 29 entries), 68.97% were confirmed as being correctly identified. Our study shows that the use of blast searches with the ITS region for identification can result in identification to a number of different species and genera. Selecting the correct placement cannot be guaranteed based on blast searches alone. Although blast searches are useful for preliminary identification of a number of pathogenic fungi, it is not suitable for accurate species resolution within *Bipolaris* as there are only a small number of reference sequences deposited in GenBank. A number of duplicate sequences in GenBank have been deposited with different species names and several species have been identified based on morphological evidence alone based on the subjective choice of phenotypic characters such as spore size and host association. It is therefore critical to use ITS blast searches only as a means for generic placement. For accurate identification in *Bipolaris*, phylogenetic analyses using multi-gene or protein coding gene such as GAPDH should be performed. It is also important to have the correct taxon sampling for accurate results, for example Xue et al. [201] included eight species of *Bipolaris* in their phylogenetic analysis which resulted in wrong identification of two of the three strains. It is therefore recommended to have the right taxon sampling with all the representative sequences generated from type material in the dataset. To support the taxon distinctiveness, there should be reliable statistical support for the clade relationship (at least 70% bootstrap or 0.90 posterior probabilities). The application of objective clustering is also recommended as additional supporting evidence for species delineation in *Bipolaris.*

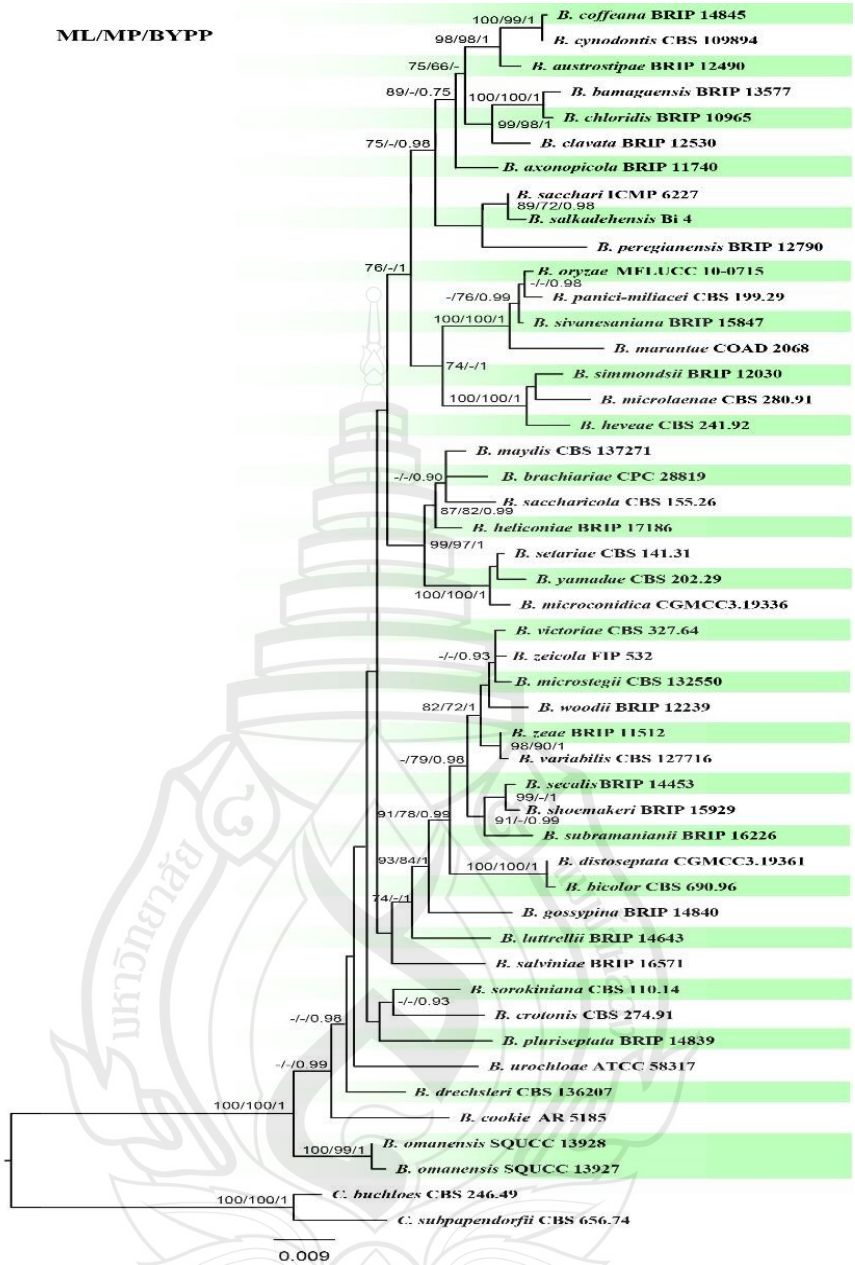
The pairwise distances using the K2P model with the “pairwise deletion” option of missing data was used to estimate the appropriate threshold for species delimitation in *Bipolaris*. The ITS and GAPDH dataset of the type sequences were used and the analysis included all codon positions and pairwise deletion of gaps for each sequence pair. The sequences of the type species that formed sister taxa in the phylogenetic tree based on the combined dataset were compared. In the ITS and GAPDH datasets, the pairwise distances varied from 0 to 2.93 and 0 to 3.996, respectively. No pairwise distance was observed in the ITS dataset between the sequences of *Bipolaris variabilis/B. zeae; B. chloridis/B. bamagaensis* and *B. cynodontis/B. coffeana*. No pairwise distance was observed in the GAPDH dataset between the sequences of *Bipolaris distoseptata/B. microconidica* and *B. sacchari/B.*

peregianensis. These species can however be differentiated based on morphological or host association. These results suggest that a specific threshold to delineate species could not be implemented and instead, species in this genus should therefore be distinguished based on a polyphasic approach, combining morphological, ecological and phylogenetic species concepts named as the Consolidated Species Concept [228].

A number of sequences formed separate clades to any type species based on our phylogenetic analyses. In the ITS tree, 13 separate clusters can be observed with statistical support (> 60% BT). A number of the species in these clusters are related to unpublished article and their placement as novel species in the genus could not be confirmed based on morphological evidence. The generic placement of these entries as novel species remains doubtful until they can be re-collected and designated a type. Some of these distinct lineages are related to published articles, however they did not have all the morphological characters for a successful comparison to the accepted species in the genus, except in Lourenço et al. [229] and Dughaishi et al. [230]. The phylogenetic tree based on the ITS sequences resulted in a cluster of *Bipolaris cactivora* species forming a sister clade to all other *Bipolaris* species with moderate statistical support, but did not cluster with the outgroup. However, sequences named as *B. cactivora* clustered with the outgroup in the GAPDH phylogenetic tree and were excluded from the analysis. Some of the *Bipolaris cactivora* entries are related to unpublished article and their placement in the genus could not be confirmed due to the lack of morphological data for comparison in the related publications. The conidia of *Bipolaris cactivora* were described as short, straight, long and 2–4-distoseptate [62], which resembles *Curvularia hawaiiensis*, however *B. cactivora* differs in having smaller conidia. The type specimen for *Bipolaris cactivora* has no molecular data available, so the generic placement of this species remains doubtful until it can be re-collected and designated a type.

In the GAPDH tree, two separate clusters (> 60% BT) to any type sequences were observed with strains described in Dughaishi et al. [230] and Lourenço et al. [229], however the statistical support was low in the ITS tree for Lourenço et al. [229]. *Bipolaris marantae* [229] is characterized by having fusiform conidia, externally thickened and truncate hila with a bipolar pattern of germination. In the original research, phylogenetic analysis based on a combined dataset of the ITS and

GAPDH dataset indicated that it is a new species which is also supported by our phylogenetic analysis of the GAPDH dataset (> 60% BT). *Bipolaris omanensis* [230] is phylogenetically closely related to *B. cookei* based on the combined dataset of ITS and GAPDH region in the original research which is also supported by our phylogenetic analysis of the ITS and GAPDH dataset (> 60% BT). *Bipolaris omanensis* differs from *B. cookei* by having shorter conidiophores, narrower conidia and a higher number of septa. *Bipolaris marantae* and *B. omanensis* are both accepted as novel species in *Bipolaris* based on morphological characters described in the original manuscripts including objective clustering result and phylogenetic support from our analysis. We therefore accept 45 species in the genus (Figure 3.11) and provide notes for all the species.



Note Phylogenetic tree generated by maximum likelihood analysis of combined ITS, GAPDH and TEF1-α sequence data of *Bipolaris* type sequences. RAxML bootstrap support and maximum parsimony values ≥ 60% (BT) as well as Bayesian posterior probabilities ≥0.90.

Figure 3.11 Phylogenetic tree based on combined dataset

3.5 Accepted Species of *Bipolaris*

1. *Bipolaris austrostipae* Y. P. Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Leyburn, from *Austrostipa verticillata* (Nees ex Spreng.) S. W. L. Jacobs & J. Everett, 11 May 1977, J. L. Alcorn; holotype: BRIP 12490 (includes ex-type culture).

Known host: *Austrostipa verticillata* [173].

Known distribution: Queensland, Australia [173].

Bipolaris austrostipae is only known from the type specimen. It is phylogenetically closely related to *B. cynodontis* and *B. coffeana* (Figure 3.11) and has overlapping conidial measurements (70–77 × 14–15.5 µm for *B. austrostipae vs.* 40–80 × 12–18 µm for *B. cynodontis vs.* 35–50 × 14–20 µm for *B. coffeana*) [172]. This species differs by 10 base pairs (out of 490 bp) from *B. cynodontis* and by 11 base pairs (out of 496 bp) from *B. coffeana* in the GAPDH gene. It differs by 1 base pair (out of 469 bp) from *B. cynodontis* in the ITS region. However, there is no base pair difference in the ITS region between *B. austrostipae* and *B. coffeana*.

2. *Bipolaris axonopicola* Y. P. Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Peregian Beach, from leaf spot on *Axonopus fissifolius* (Raddi) Kuhlm., 6 June 1976, J. L. Alcorn; holotype: BRIP 11740 (includes ex-type culture).

Known host: *Axonopus fissifolius* [173].

Known distribution: south-east Queensland, Australia [173].

Bipolaris axonopicola is only known from the type specimen. *Bipolaris axonopicola* and *B. cynodontis* have overlapping conidial measurements (55–60 × 11.5–12.5 µm in *B. axonopicola vs.* 40–80 × 12–18 µm in *B. cynodontis*). It has straight conidia which distinguishes it from *B. cynodontis* [173]. *Bipolaris axonopicola* forms a separate lineage to *B. coffeana, B. cynodontis, B. austrostipae, B. bamagaensis* and *B. chloridis* (Figure 3.11). This species differs by 18 base pairs (out of 490 bp) from *B. cynodontis,* 14 base pairs (out of 496 bp) from *B. austrostipae,* 19

base pairs (out of 491 bp) from *B. bamagaensis* and by 18 base pairs (out of 496 bp) from *B. chloridis* in the GAPDH gene region. It differs by 2 base pairs (out of 396 bp) from *B. coffeana,* 6 base pairs (out of 469 bp) from *B. cynodontis,* 7 base pairs (out of 469 bp) from *B. austrostipae,* 11 base pairs (out of 470 bp) from *B. bamagaensis* and 5 base pairs (out of 397 bp) from *B. chloridis* in the ITS region.

3. *Bipolaris bamagaensis* Y. P. Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Bamaga, from necrotic leaf on *Urochloa subquadripara* (Trin.) R. D. Webster, 28 May 1981, J. L. Alcorn; holotype: BRIP 13577 (includes ex-type culture).

Known hosts: *Brachiaria subquadripara*, *Dactyloctenium aegyptium* [173].

Known distribution: Northern Queensland, Australia [173].

The hosts *Dactyloctenium aegyptium* and *Urochloa subquadripara* are found across Australia, however *Bipolaris bamagaensis* has only been found in northern Australia [173]. Several *Bipolaris* species have been associated with the host *Dactyloctenium*, including *Bipolaris clavata, B. cynodontis, B. luttrellii* and *B. maydis* whereas only *B. urochloae* has been recorded on *Urochloa* [171,172]. This species is phylogenetically closely related to *B. chloridis* (Figure 3.11). It differs by having shorter conidiophores in culture compared to *B. chloridis* (up to 1.2 mm long) [173]. *Bipolaris bamagaensis* differs by 3 base pairs (out of 491 bp) from *B. chloridis* in the GAPDH gene. However, there is no base pair difference in the ITS region between *B. bamagaensis* and *B. chloridis*.

4. *Bipolaris bicolor* (Mitra) Shoemaker, Canad. J. Bot. 37: 884. [165].

Type material: India, Poona, associated with wheat foot-rot; Australia, Queensland, and New South Wales, Bega, dry culture on Sach's agar + pieces of *Danthonia spikelet* and pedicel, produced by pairing two isolates from *Pennisetum clandestinum* from Toowoomba, Queensland, Australia, 1966, A. R. Paul & Parbery, MELU F2220 (*Cochliobolus bicolor*).

Known hosts: *Eleusine indica* [231]; *Hevea brasiliensis* [232]; (*Andropogon aciculatus, Apluda aristata, Brachiaria ruziziensis, Eleusine coracana, Eragrostis japonica, Melanocenchris abyssinica, Oryza sativa, Panicum maximum, Pennisetum clandestinum, P. glaucum, P. typhoides, Quercus* sp., *Setaria* sp.,

Sorghum sp., *S. vulgare, Triticum aestivum, Urochloa panicoides, Zea mays*., *Zizania aquatica*) [233].

Known distribution: Australia [172]; China [232]; Thailand [231]; (Africa, Brazil, Canada, Cote d'Ivoire, Denmark, India, New Zealand, Nigeria, Swaziland, Zimbabwe) [233].

Bipolaris bicolor is relatively common in warm temperate and tropical regions. It is considered to be a seed-borne pathogen [234] and it has been associated with foot rot of wheat and leaf spot of *Pennisetum* [171]. There is no available ex-type culture for this species and the culture CBS 690.96 is presently used as the representative strain. This species is phylogenetically closely related to *B. distoseptata* (Figure 3.11). It differs by 1 base pair (out of 462 bp) from *B. distoseptata* in the ITS region. However, there is no base pair difference in the GAPDH gene between *B. bicolor* and *B. distoseptata*.

5. *Bipolaris brachiariae* Y. Marín, Senwanna & Crous, Mycosphere. 8: 1561. [231].

Type material: Thailand, Chiang Mai, Mae Hia Agricultural Research, Demonstrative and Training Center, on *Brachiaria mutica*, 28 August 2010, C. Senwanna; holotype: CBS H-23191; ex-type culture: CPC 28819.

Known host: *Brachiaria mutica* [231].

Known distribution: Thailand [231].

Bipolaris brachiariae is phylogenetically closely related to *B. maydis* and *B. saccharicola* (Figure 3.11). This species is distinguishable based on the size of the conidiophores (up to 195 µm long in *Bipolaris brachiariae vs.* 712 µm long in *B. maydis vs.* 900 µm long in *B. saccharicola*), and conidia (20–87.5 µm long in *B. brachiariae vs.* 66–102 µm long in *B. maydis vs.* 45–120 µm long in *B. saccharicola*) [231]. It differs by 7 base pairs (out of 490 bp) from *B. maydis* and by 7 base pairs (out of 496 bp) from *B. saccharicola* in the GAPDH gene region. *Bipolaris brachiariae* differs by 14 base pairs (out of 475 bp) from *B. maydis* and by 14 base pairs (out of 470 bp) from *B. saccharicola* in the ITS region.

6. *Bipolaris chloridis* (Alcorn) Alcorn, Mycotaxon 16: 373. [172].

Type material: Australia, Queensland, Booie prop, Kingaroy, on *Chloris gayana*, 21 Dec. 1972, J. L. Alcorn; holotype: BRIP 10965 (*Bipolaris chloridis*) (includes ex-type culture); isotype: IMI 181067.

Known host: *Chloris gayana* (*Poaceae*) [172].

Known distribution: Australia [172]; (India, Kenya, Malawi, Tanzania, Zambia) [233].

Bipolaris chloridis has similar conidial morphology to *B. cookei* in terms of dimensions and septation. *Bipolaris cookei* often produces secondary conidiophores and secondary conidia which are not present in *B. chloridis* [171,172]. *Bipolaris chloridis* and *B. cookei* are not phylogenetically closely related. This species is phylogenetically closely related to *B. bamagaensis*. *Bipolaris chloridis* differs by 3 base pairs (out of 491 bp) from *B. bamagaensis* in the GAPDH gene. However, there is no base pair difference in the ITS region between *B. bamagaensis* and *B. chloridis*. The holotype (BRIP 10965) for this species is not available, so the isotype BRIP 12106a is used as the representative strain.

7. *Bipolaris clavata* Alcorn, Mycotaxon 15: 15. [235].

Type material: Australia, Queensland, on *Dactyloctenium radulans*, 12 May 1977, J. L. Alcorn 77144C; holotype: BRIP 12530 (includes ex-type culture); ex-holotype culture: Alcorn 77144c.

Known host: *Dactyloctenium radulans* (Poaceae) [173].

Known distribution: Australia [173].

This species is only known from the type specimen. Alcorn [235] reported conidial dimorphism in *Bipolaris clavata*. The ex-type culture of this species produced two types of conidia, specifically large, curved-clavate conidia typical of *Bipolaris* and small cylindrical conidia. The conidial dimorphism is a unique character of this species but it has not been observed with the isotypes [172]. *Bipolaris clavata* is phylogenetically closely related to *B. chloridis* and *B. bamagaensis* (Figure 3.11). It differs by 6 base pairs (out of 496 bp) from *B. chloridis* and by 7 base pairs (out of 491 bp) from *B. bamagaensis* in the GAPDH gene region. This species differs by 8 base pairs (out of 398 bp) from *B. chloridis* and by 8 base

pairs (out of 398 bp) from *B. bamagaensis* in the ITS region A sexual morph has not been described for this species.

8. *Bipolaris coffeana* Sivan., Trans. Brit. Mycol. Soc. 84: 404. [236].

Type material: Kenya, on *Coffea arabica*, 31 Oct. 1969, I. Furtado; holotype: IMI 144159; ex-isotype: BRIP 14845.

Known hosts: *Bouteloua gracilis, Cynodon dactylon* and *Digitaria* sp. (*Poaceae*) [172]; on leaves of *Coffea arabica* (*Rubiaceae*) [173].

Known distribution: Kenya [173]; New Zealand, Thailand, USA [172].

Bipolaris coffeana is only known from the type specimen. *Bipolaris tropicalis* and *B. zeicola* have also been reported on *Coffea*, but *B. coffeana* differs by having smaller conidia compared to *B. zeicola* (65–90 μm). Stromata production was observed in the culture of *Bipolaris tropicalis* unlike in *B. coffeana*. Conidial germination has only been described by Sivanesan [236]. *Bipolaris coffeana* is phylogenetically closely related to *B. cynodontis* (Figure 3.11). This species differs by 1 base pair (out of 490 bp) from *B. cynodontis* in the GAPDH gene region. However, there is no base pair difference in the ITS region between *B. coffeana* and *B. cynodontis*. A sexual morph has not been described for this species.

9. *Bipolaris cookei* (Sacc.) Shoemaker, Canad. J. Bot. 37: 884. [165].

Type material: USA, South Carolina, Aiken, on culms of *Sorghum* sp., (1874) Ravenel, American Fungi no. 167; holotype: BPI 430300 (*Helminthosporium cookei*); paratypes: BPI 430369.

Known hosts: *Sorghum bicolor, S. halapense, Sorghum* sp., *S. sudanense, S. vulgare, Zea mays* [172]; (*Chloris gayana, Eriochloa procera, Oryza sativa*) [233].

Known distribution: Japan, USA [172]; (Australia, Bolivia, Brazil, China, Cuba, Guyana, India, Korea, Malaysia, Nigeria, Pakistan, Papua New Guinea, Saudi Arabia, Solomon Islands, Sri Lanka, Sudan, Taiwan, Togo, Yemen, Zimbabwe) [233].

Bipolaris cookei has been associated with serious loss in the production of *Sorghum*. Manamgoda et al. [172] observed specimens of *Bipolaris cookei* and concluded that it is morphologically and symptomatically similar to *B. sorghicola*. This is in agreement to previous study by Saccardo [237] which concluded that *B. sorghicola* is a synonym of *B. cookei*. This species forms a distinct lineage to other species (Figure 3.11). A sexual morph has not been described for this species.

10. *Bipolaris crotonis* Sivan., Trans. Brit. Mycol. Soc. 84: 404. [236].

Type material: Australia, Queensland, Goldsborough, from leaf spot of *Eleusine indica*, 1 May 1987, J. L. Alcorn 8786a; holotype: BRIP 15875 (*Bipolaris eleusines* Alcorn & R. G. Shivas); ex-isotype culture: CBS 274.91.

Known hosts: On decaying leaves of *Croton* sp. (*Euphorbiaceae*), *Eleusine indica* (*Poaceae*) [173].

Known distribution: Australia [173]; Samoa [172].

Tan et al. [173] determined that the ex-isotype of *Bipolaris crotonis* (BRIP 14838) and the ex-type isolate of *B. eleusines* (BRIP 15875) [238] are identical based on phylogenetic analyses. They are also morphologically similar in having slightly protruding and truncated hila with similar conidial measurements (80–110×18–29 μm for *B. crotonis vs.* 75–170×15–26 μm for *B. eleusines*) [173]. *Bipolaris crotonis* is closely related to *B. sorokiniana* and *B. pluriseptata* (Figure 3.11). It differs by 14 base pairs (out of 490 bp) from *B. sorokiniana* and by 22 base pairs (out of 492 bp) from *B. pluriseptata* in the GAPDH gene region. It differs by 2 base pairs (out of 463 bp) from *B. sorokiniana* and by 10 base pairs (out of 399 bp) from *B. pluriseptata* in the ITS region.

11. *Bipolaris cynodontis* (Marignoni) Shoemaker, Canad. J. Bot. 37: 883. [165].

Type material: Italy, on *Cynodon dactylon*, Micromiceti di Schio: 27, J.A. Stevenson Mycology Library, USDA-ARS, Beltsville, Maryland, USA (*Helminthosporium cynodontis*) “MBT197968”. Hungary, Keszthely, on *Cynodon dactylon*, 1992, J. Bakonyim; epitype: BPI 892949; dried culture specimen: “MBT198051” (*Bipolaris cynodontis*); ex-epitype culture: CBS 109894. USA, dried culture on Sach's agar with *Zea mays*, Nov. 1962, R. R. Nelson 85101; holotype: BPI 626389 (*Cochliobolus cynodontis*).

Known hosts: *Cynodon dactylon, Echinochloa crus-galli, Eragrostis pectinacean, Miscanthus sinensis, Muhlenbergia mexicana, Panicum philadelphicum, Zea mays* (*Poaceae*) [172]; *Cynodon transvaalensis* [204]; (*Arthraxon affinis, A. hispidus, Brachiaria brizantha, B. platyphylla, Cardiospermum corindum*, *Cynodon bradleyi, C. plectostachyus, Cynosurus cristatus, Dactylis glomerata, Dactyloctenium aegyptium, Eleusine indica, Elymus riparius, Eragrostis pectinacea, Eucalyptus* sp.,

Festuca sp., *Heteropogon contortus, Hordeum* sp., *Hordeum vulgare, Leptochloa fascicularis, Ligustrum lucidum, Lolium multiflorum, L. × multiflorum-perenne, Microstegium vimineum, Muhlenbergia schreberi, M. sylvatica, M. tenuiflora, Oryza sativa, Panicum maximum, Paspalum conjugatum, Pennisetum clandestinum, P. purpureum, P. typhoides, Phyllostachys* sp., *Pinus caribaea, Rosa* sp., *Saccharum officinarum, Secale cereal, Senecio mesogrammoides, Setaria geniculate, S. glauca, S. pumila, Sorghum arundinaceum, S. halepense, Triticum* sp.) [233].

Known distribution: Hungary, Italy, USA [172]; Kenya [204]; New Zealand [180]; (Argentina, Australia, Bangladesh, Brazil, Brunei Darussalam, Ghana, Guinea, India, Malaysia, Myanmar, New Guinea, Nicaragua, Pakistan, Papua New Guinea, South Africa, Tanzania, Thailand, Turkey, Venezuela, Yugoslavia, Zambia, Zimbabwe) [233].

Bipolaris cynodontis can be a pathogen, secondary invader or saprobe on a wide range of hosts [172]. A strain of this species produces the phytotoxin bipolaroxin. There is considerable variation in the conidial and conidiophore measurements of *Bipolaris cynodontis.* This species has the unique feature of swollen end cells of germinating spores. It is phylogenetically closely related to *B. coffeana* (Figure 3.11). This species differs by 1 base pair (out of 490 bp) from *B. coffeana* in the GAPDH gene region. However, there is no base pair difference in the ITS region between *B. cynodontis* and *B. coffeana* in the ITS region.

12. *Bipolaris distoseptata* M. Raza, K. D. Hyde & L. Cai, sp. nov., Fungal Divers 99:1. [187].

Type material: China, Guangxi Province, Guilin City, Yangshuo County, on *Saccharum officinarum*, Aug. 2016, M. Raza and Y. Z. Diao; holotype: HMAS 248049; ex-holotype living culture: CGMCC 3.19361 = LC12045.

Known host: *Saccharum officinarum* [187].

Known distribution: China [187].

Bipolaris distoseptata is phylogenetically closely related to *B. bicolor* (Figure 3.11). This species has been isolated from *Saccharum officinarum,* whereas *B. bicolor* has been associated with a number of hosts except *S. officinarum.* It differs from *B. bicolor* by having smaller conidiophores (40–80 × 5–8 µm *vs.* 150–490 × 5–7 µm in *B. bicolor*) [187]. This species produces ellipsoid, oblong or cylindrical conidia

compared to the curved, cylindrical or obclavate conidia of *B. bicolor*. A detailed description of this species is available in Raza et al. [187]. It differs by 1 base pair (out of 462 bp) from *B. bicolor* in the ITS region. However, there is no base pair difference in the GAPDH gene between *B. bicolor* and *B. distoseptata*. A sexual morph has not been described for this species.

13. *Bipolaris drechsleri* Manamgoda & Minnis., Persoonia 31: 293. [172].

between *B. cynodontis* and *B. coffeana* in the ITS region.

Type material: USA, Indiana, Big Oaks Wildlife Refuge, on living leaves of *Microstegium vimineum*, 2010, N. Kleczewski; holotype: BPI 892682; ex-holotype culture: CBS 136207.

Known host: *Microstegium vimineum* [172].

Known distribution: USA [172].

Bipolaris drechsleri and *B. microstegii* are associated with *Microstegium vimineum* in the USA. Both species have similar conidial dimensions (50–80 × 13–19 µm *vs.* 45–86 × 14–18 µm in *B. microstegii*), but *Bipolaris drechsleri* differ by having shorter conidiophores (95–300 × 4–6 µm *vs.* 300–700 × 7–9 µm in *B. microstegii*) with more proliferations [172]. This species forms a separate lineage to other *Bipolaris* species (Figure 3.11). There is no sexual morph associated with this species.

14. *Bipolaris gossypina* Sivan., Trans. Brit. Mycol. Soc. 84: 404. [236].

Type material: Kenya, on *Gossypium* sp., 1966, W. H. White; holotype: BRIP 14840 (includes ex-type culture).

Known host: *Gossypium* sp. [173].

Known distribution: Kenya [173].

Bipolaris gossypina is only known from the type specimen and it is the only *Bipolaris* species recorded on *Gossypium*. The only other species that resembles *Bipolaris* that has been recorded on *Gossypium* is *Helminthosporium gossypii*. This species differs from *Bipolaris gossypina* in shape and bipolar germination [172]. *Bipolaris gossypina* forms a separate lineage to other *Bipolaris* species (Figure 3.11). There is no sexual morph associated with this species.

15. *Bipolaris heliconiae* Alcorn, Austral. Syst. Bot. 9: 814. [235].

Type material: Australia, Northern Territory, Batchelor, on *Heliconia psittacorum*; holotype: BRIP 17186 (*Bipolaris heliconiae*) (includes ex-type culture);

on *Heliconia* sp., 18 Jan. 1991, J. L. Alcorn; holotype: BRIP 17349 (*Cochliobolus heliconiae*).

Known hosts: *Heliconia chartacea*, *Heliconia psittacorum* [173]; *Heliconia* sp. [172].

Known distribution: Australia [173].

A number of *Bipolaris* species have been found on the host *Heliconia* including *Bipolaris heliconiae, B. cynodontis, B. salviniae* and *B. setariae*. *Bipolaris cynodontis* and *B. setariae* differ from *B. heliconiae* by their smaller spores (40–80 × 12–18 µm in *B. cynodontis vs.* 65–100 × 13–16 µm in *B. setariae vs.* 65–150 × 15–19 µm in *B. heliconiae*) [172]. *Bipolaris heliconiae* produces fusoid to clavate conidia whereas *B. salviniae* produces cylindrical conidia. This species is phylogenetically closely related to *Bipolaris brachiariae, B. maydis* and *B. saccharicola* (Figure 3.11). It differs by 8 base pairs (out of 496 bp) from *B. brachiariae,* 5 base pairs (out of 490 bp) from *B. maydis* and by 5 base pairs (out of 496 bp) from *B. saccharicola* in the GAPDH gene region. This species differs by 9 base pairs (out of 402 bp) from *B. brachiariae,* 6 base pairs (out of 400 bp) from *B. maydis* and by 6 base pairs (out of 402 bp) from *B. saccharicola* in the ITS region.

16. *Bipolaris heveae* (Petch) Arx, Nova Hedwigia, Beih. 87: 288. [172].

Type material: Sri Lanka, Gampaha, Henarathgoda, on *Hevea brasiliensis*, Mar. 1917, T. Petch 5030; holotype: K(M) 181465.

Known host: *Hevea brasiliensis* [172].

Known distribution: Cambodia, Dominican Republic, Ghana, Guatemala, Haiti, Honduras, Indonesia, Mexico, Nigeria, Philippines, Sri Lanka, USA [172].

Bipolaris heveae has been associated with leaf spots on *Hevea brasiliensis* in rubber growing countries in the tropics [172]. *Bipolaris heveae* is phylogenetically closely related to *B. microlaenae* and *B. simmondsii* (Figure 3.11). *Bipolaris heveae* differs from *B. microlaenae* as a sexual morph has been recorded for *B. microlaenae* unlike in *B. heveae*. *Bipolaris microlaenae* has only been reported from Australia unlike *B. heveae* which has been found in the tropics. This species differs by 13 base pairs (out of 490 bp) from *B. microlaenae* and by 10 base pairs (out of 490 bp) from *B. simmondsii* in the GAPDH gene region. It differs by 3 base pairs (out of 475 bp)

from *B. microlaenae* and by 3 base pairs (out of 475 bp) from *B. simmondsii* in the ITS region.

17. *Bipolaris luttrellii* Alcorn, Mycotaxon 39: 378. [238]

Type material: Australia, on *Dactyloctenium aegyptium*, 3 Jun. 1985, J. L. Alcorn; dried culture holotype: BRIP 14791 (*Cochliobolus luttrellii*); isotype: IMI 335215, Northern Territory, on *Dactyloctenium aegyptium*, 31 Mar. 1985, R. A. Peterson; holotype: BRIP 14643 (*Bipolaris luttrelii*); isotype: IMI 332216.

Known host: *Dactyloctenium aegyptium* [172].

Known distribution: Australia [172].

Bipolaris luttrellii is only known from the type specimen. It is morphologically similar to *Bipolaris setariae* but it differs by having fewer conidiogenous loci on the conidiophores [172]. *Bipolaris luttrellii* also differs by its darker conidia with pale end cells compared to the concolorous conidia of *B. setariae*. This species forms a distinct lineage to other species (Figure 3.11).

18. *Bipolaris marantae* J. L. Alves & R. W. Barreto sp. nov., Mycobiology. 45:3. [229].

Type material: Brazil, Rio de Janeiro, Gávea on leaves of *Maranta leuconeura*, 10 Nov 2015, Robert W. Barreto; holotype: VIC 44075; ex-type culture: COAD 2068.

Known host: *Maranta leuconeura* [229].

Known distribution: Brazil [229].

Bipolaris marantae has similar morphology to *B. setariae* and *B. oryzae*. It differs from *B. setariae* by its larger conidia (45–100 × 10–15 µm *vs*. 80–150 × 12.5–22.5 µm in *B. marantae*). This species differs from *Bipolaris oryzae* by its longer conidia (63–153 × 14–22 µm in *B. oryzae*) and by conidial pigmentation (pale to mid-golden brown in *B. oryzae* and pale to dark brown in *B. marantae*) [229]. *Bipolaris marantae* is phylogenetically closely related to *Bipolaris panici-miliacei, B. oryzae* and *B. sivanesaniana* (Figure 3.11). This species differs by 6 base pairs (out of 338 bp) from *B. panici-miliacei*, by 7 base pairs (out of 338 bp) from *B. oryzae* and by 7 base pairs (out of 334 bp) from *B. sivanesaniana* in the GAPDH gene region. It differs by 19 base pairs (out of 489 bp) from *B. panici-miliacei*, by 22 base pairs (out

of 489 bp) from *B. oryzae* and by 21 base pairs (out of 490 bp) from *B. sivanesaniana* in the ITS region. A sexual morph has not been described for this species.

19. *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker, Canad. J. Bot. 33: 882. [165].

Type material: USA, North Carolina, isolated from *Zea mays*, Olin Yoder C5; neotype: dried culture ATCC 48332; ex-neotype culture: CBS 137271; Florida, Sanford, on *Z. mays*, 22 Sep. 1923, C. Drechsler; holotype: BPI 626700 (*Cochliobolus heterostrophus*).

Known hosts: *Sorghum bicolor, Zea mays*; *Vitis vinifera* [172]; (*Antirrhinum majus, Bothriochloa insculpta, Brachiaria foliosa, Chloris gayana, C. virgate, Coix lacryma-jobi, Cymbopogon citratus, C. martini, Cynodon dactylon, Dactyloctenium aegyptium, Dianthus caryophyllus, Digitaria ciliaris, Echinochloa colonum, E. crus-galli, Euchlaena mexicana, Eleusine indica, Eriochloa procera, Oryza sativa, Panicum bisulcatum, P. maximum, P. miliaceum, P. palmifolium, Paspalum scrobiculatum, Pennisetum maximum, P. typhoides, Perotis indica, Populus deltoids, Rottboellia exaltata, Saccharum officinarum, Salacca wallichiana, Setaria barbata, S. viridis, S. homonyma, S. sphacelata, Sorghum halepense, S. vulgare, Sorghum* sp., *Sporobolus poiretiana, Triticum* sp.) [233].

Known distribution: China, Japan, USA [172]; Thailand [231]; (Australia, Bahamas, Bhutan, Bolivia, Brazil, Brunei Darussalam, Denmark, Egypt, Gambia, Ghana, Hong Kong, India, Jamaica, Malawi, Malaysia, Myanmar, Nepal, New Zealand, Nicaragua, Nigeria, Pakistan, Papua New Guinea, Portugal, Samoa, Sierra Leone, Solomon Islands, South Africa, Sudan, Swaziland, Taiwan, Trinidad, Tobago, Zambia) [233].

Bipolaris maydis is an economically important pathogen responsible for southern leaf blight of maize [171]. The conservation of the basionym *Helminthosporium maydis* was proposed over the two other synonyms *Helminthosporium maydis* Brond. and *Ophiobolus heterostrophus* Drechsler as it is more commonly used in literature. *Bipolaris maydis* is phylogenetically closely related to *B. brachiariae* and *B. saccharicola* (Figure 3.11). It differs by 7 base pairs (out of 490 bp) from *B. brachiariae* and by 7 base pairs (out of 496 bp) from *B. saccharicola* in the GAPDH gene region. *Bipolaris maydis* differs by 14 base pairs

(out of 475 bp) from *B. brachiariae* and by 14 base pairs (out of 470 bp) from *B. saccharicola* in the ITS region.

20. *Bipolaris microconidica* M. Raza, K. D. Hyde & L. Cai, sp. nov., Fungal Divers 99:1. [187].

Type material: China, Guangxi Province, Liuzhou City, Liqing County, on *Saccharum officinarum*, Aug. 2016, M. Raza and Y.Z. Diao; holotype: HMAS 248047; ex-holotype living culture CGMCC 3.19336 = LC12024.

Known host: *Saccharum officinarum* [187].

Known distribution: China [187].

Bipolaris microconidica is phylogenetically closely related to *B. setariae* and *B. yamadae* (Figure 3.11). It differs based on conidial measurements from *B. setariae* (35–58 × 10–15 *vs.* 65–100 × 13–16 μm in *B. setariae*) and *B. yamadae* (65–100 × 14–18 μm) [187]. This species differs by its cylindrical, ellipsoidal, or obclavate conidia with obtuse ends compared to the fusoid, fusiform, obclavate or obovoid conidia with rounded ends of *Bipolaris setariae* and *B. yamadae*. Microconidiation was observed in *Bipolaris microconidica* unlike in *B. setariae* and *B. yamadae*. It differs by 2 base pairs (out of 490 bp) from *Bipolaris yamadae* in the GAPDH gene region. However, there is no base pair difference in the GAPDH gene between *B. microconidica* and *B. setariae*. This species differs by 9 base pairs (out of 474 bp) from *B. yamadae* and by 7 base pairs (out of 474 bp) from *B. setariae* in the ITS region. A sexual morph has not been described for this species.

21. *Bipolaris microlaenae* Alcorn, Mycotaxon 39: 382. [238].

Type material: Australia, Queensland, Highfields, on *Microlaena stipoides*, 4 Mar. 1987, J. L. Alcorn 8705; holotype: BRIP 15613 (*Bipolaris microlaenae*); culture ex-type: CBS 280.91.

Known host: *Microlaena stipoides* [178].

Known distribution: Australia [178].

Bipolaris microlaenae differs from other *Bipolaris* species found in Australia based on morphological characters of both sexual and asexual morphs [238]. This species is phylogenetically closely related to *B. heveae* and *B. simmondsii* (Figure 3.11). It differs from *B. heveae* which is only known from the asexual morph. *Bipolaris microlaenae* is only known from Australia whereas *B. heveae* has been

reported from tropical countries. This species differs by 13 base pairs (out of 490 bp) from *B. heveae* and by 10 base pairs (out of 490 bp) from *B. simmondsii* in the GAPDH gene region. It differs by 3 base pairs (out of 475 bp) from *B. heveae* and by 3 base pairs (out of 475 bp) from *B. simmondsii* in the ITS region.

22. *Bipolaris microstegii* Minnis et al., Persoonia 29: 151. 2012.

Type material: USA, West Virginia, near Arnoldsburg, Crummies Creek Tree Farm, on living leaves of *Microstegium vimineum*, Aug. 2009, R. Richardson; holotype: BPI 883727; ex-type culture: CBS 132550.

Known host: *Microstegium vimineum* [172].

Known distribution: USA [172].

Several *Bipolaris* species are associated with *Microstegium* in Asia, but the origin of *B. microstegii* is unknown [172]. *Bipolaris microstegii* is phylogenetically closely related to the plant pathogens *B. victoriae* and *B. zeicola* (Figure 3.11). This species differs by 1 base pair (out of 490 bp) from *Bipolaris victoriae* and *B. zeicola* in the GAPDH gene region. It differs by 2 base pairs (out of 469 bp) from *Bipolaris victoriae* and by 5 base pairs (out of 464 bp) from *B. zeicola* in the ITS region. A sexual morph has not been described for this species.

23. *Bipolaris omanensis* Dughaishi, Maharachch. & Al-Sadi, sp. nov., Phytotaxa. 385: 1. [230].

Type material: OMAN, Muscat: Sultan Qaboos University Botanical Garden, on leaves of *Hibiscus rosa-sinensis* (Malvaceae), 25 May 2017, SA Dughaishi; holotype SQU H-102; ex-type culture: SQUCC 13828.

Known host: *Hibiscus rosa-sinensis* [230].

Known distribution: Oman [230].

This species has been isolated from the leaves of *Hibiscus rosa-sinensis* in Oman. *Bipolaris omanensis* and *B. gossypina* are associated with hosts from the family Malvaceae [171]. *Bipolaris gossypina* differs by its smaller conidia (74–129 × 14–17 *vs.* 40–70 × 15–21 µm in *B. gossypina*) [171]. This species forms a distinct lineage to other species (Figure 3.11). A sexual morph has not been described for this species.

24. *Bipolaris oryzae* (Breda de Haan) Shoemaker, Canad. J. Bot. 37: 883. [165].

Type material: Japan, on *Oryza sativa*, Ito & Kuribayashi (1927), Ann. Phytopathol. Soc. Japan 2: 9 plate I, J.A. Stevenson Mycology Library, USDA-ARS, Beltsville, Maryland, USA (*Ophiobolus miyabeanus*) "MBT197970". Thailand, Chiang Rai, near Khunkoon waterfall, on seeds of Oryza *sativa*, May 2010, D.S. Manamgoda; neotype: BPI 892948 (*Bipolaris oryzae*); ex-neotype culture: MFLUCC 10-0715.

Known hosts: *Oryza australiensis, Oryza sativa, Panicum virgatum* [172]; (*Alopecurus aequalis*, *Chikusichloa aquatic*, *Cordia trichotoma*, *Eleusine indica*, *Leersia hexandra*, *Oxalis latifolia*, *Panicum colonum, P. maximum, P. virgatum*, *Setaria italica, Triticum aestivum, Zizania latifolia, Z. palustris*) [233].

Known distribution: Australia, Thailand, USA [172]; (Bangladesh, Bhutan, Bolivia, Brazil, Brunei Darussalam, China, Colombia, Egypt, Fiji, Gambia, Ghana, Guinea, India, Indonesia, Iran, Jamaica, Japan, Korea, Malawi, Malaysia, Mauritius, Mexico, Myanmar, Nepal, New Zealand, Nicaragua, Nigeria, Pakistan, Panama, Papua New Guinea, South Africa, Venezuela, Yugoslavia, Zambia, Zimbabwe) [233].

Bipolaris oryzae was the causative agent of devastating diseases resulting in the 1943 Bengal famine in India [56]. Manamgoda et al. [172] designated a neotype from Thailand as the type specimen could not be found. There is considerable variation in conidial morphology [172] and considerable genetic variation within the species (Cholil and de Hoog 1982). It is phylogenetically closely related to *Bipolaris panici-miliacei* (Figure 3.11). This species differs from *B. panici-miliacei* by 3 base pairs (out of 489 bp) in the GAPDH gene region and by 2 base pairs (out of 473 bp) in the ITS region.

25. *Bipolaris panici-miliacei* (Y. Nisik.) Shoemaker, Canad. J. Bot. 37: 883. [165].

Type material: Japan, on *Panicum miliaceum*, Y. Nisikado, Ber. Ohara Inst. Landw. Fursch. 4:120 (1929): plate XIII, J. A. Stevenson Mycology Library, USDA-ARS, Beltsville, Maryland. USA, on *Panicum miliaceum*, Nov. 1929, Y. Nisikado; lectotype: CBS H7031; ex-lectotype culture: CBS 199.29 "MBT197971".

Known hosts: *Panicum miliaceum* (*Poaceae*) [172]; (*Brachiaria foliosa. Panicum psilopodium, Setaria palmifolia*) [233].

Known distribution: Japan [172]; (Australia, India, New Guinea) [233].

Manamgoda et al. [172] designated a lectotype from available syntypes with a culture as the type specimen could not be found. *Bipolaris panici-miliacei* is morphologically similar to *B. oryzae* and they are phylogenetically closely related to each other (Figure 3.11). *Bipolaris panici-miliacei* differs from *B. oryzae* by 3 base pairs (out of 489 bp) in the GAPDH gene region and by 2 base pairs (out of 473 bp) in the ITS region.

26. *Bipolaris peregianensis* Alcorn, Mycotaxon 15: 9. [235].

Type material: Australia, Queensland, Oriunda, Perigian Beach, on leaf of *Cynodon dactylon*, 11 Nov. 1978, J. L. Alcorn 7848; holotype: BRIP 12790; ex-holotype culture: IMI 264355.

Known hosts: *Cynodon dactylon* [172]; *C. dactylon* × *C. transvaalensis* [225]; (*Rottboellia exaltata*) [233].

Known distribution: Australia [172]; China [225].

Bipolaris peregianensis and *B. cynodontis* are associated with the host *Cynodon dactylon.* This species has been reported to be responsible for small, elliptical, pale brown lesions. It is phylogenetically closely related to *B. sacchari* and *B. salkadehensis* (Figure 3.11). This species differs by 18 base pairs (out of 466 bp) from *Bipolaris sacchari* and by 20 base pairs (out of 467 bp) from *B. salkadehensis* in the ITS region. However, there is no base pair difference in the GAPDH gene between *Bipolaris peregianensis* and *B. sacchari.* The GAPDH gene region of *Bipolaris salkadehensis* is not available for comparison.

27. *Bipolaris pluriseptata* (Khetarpal, R. Nath & S. P. Lal) Alcorn, Mycotaxon 41: 329. 1991.

Type material: Zambia, on seeds of *Eleusine coracana*, Feb. 1981; holotype: IMI 259810; ex-isotype culture: BRIP 14839.

Known hosts: *Eleusine coracana* [173]; (*Sorghum bicolor*) (Farr and Rossman 2020).

Known distribution: Zambia [173]; (India, Saudi Arabia) [233].

Bipolaris pluriseptata has the longest conidia in the genus (up to 300 μm) compared to an average of about 100 μm [172]. This species has strongly curved conidia. It is phylogenetically closely related to *B. sorokiniana* and *B. crotonis* (Figure 3.11). It differs by 25 base pairs (out of 492 bp) from *B. sorokiniana* and by 22 base pairs (out of 492 bp) from *B. crotonis* in the GAPDH gene region. It differs by 11 base pairs (out of 400 bp) from *B. sorokiniana* and by 11 base pairs (out of 399 bp) from *B. crotonis* in the ITS region. A sexual morph has not been described for this species.

28. *Bipolaris sacchari* (E.J. Butler) Shoemaker, Canad. J. Bot. 17: 68. [165].

Type material: India, Pusa, on *Saccharum officinarum*, collection details unknown (*Bipolaris sacchari*) [171]; Cuba, on *Saccharum officinarum*, 1927, J. A. Faris; holotype: ICMP 6227 (includes ex-type culture); lectotype: BPI 429720 (*Helminthosporium ocellum*).

Known hosts: *Oplismenus imbecillus, Pennisetum purpureum, Saccharum officinarum* [172]; (*Cunninghamia lanceolate, Cymbopogon citratus, C. citrates, Cynodon dactylon, Digitaria insularis, Echinochloa colona, Imperata arundinacea, I. cylindrica, Iseilema laxum, Leptocoryphium lanatum, Lygodium japonicum, L. microphyllum, Musa paradisiaca, Panicum fasciculatum, P. maximum, P. purpurascens, Pennisetum clandestinum, P. glaucum, P. typhoides, Saccharum* sp.*, Tillandsia* sp.*, Triticum aestivum, Zea mays*) [233].

Known distribution: Brazil, Cook Islands, Cuba, India, New Zealand, Philippines, Puerto Rico, Uganda, USA, [172]; (Australia, China, Costa Rica, Dominican Republic, El Salvador, Fiji, Guatemala, Honduras, Indonesia, Iran, Italy, Jamaica, Malawi, Malaysia, Myanmar, Nicaragua, Nigeria, Panama, Papua New Guinea, Senegal, Sierra Leone, Solomon Islands, Southern Africa, Sri Lanka, Taiwan, Trinidad, Venezuela, West Indies) [233].

Helminthosporium sacchari was associated with eyespot disease on sugar cane in India [239]. Subramanian [240] synonymised *Helminthosporium sacchari* with *H. ocellum*, and placed it in *Bipolaris*. A species deposited in BPI (BPI 429720) [241] was later designated as the lectotype for *Helminthosporium ocellum* by R.A. Shoemaker. Manamgoda et al. [172] found that *Helminthosporium sacchari* and *H. ocellum* are conspecific based on morphological data and the oldest epithet sacchari

has priority, so the current species name is *Bipolaris sacchari*. *Bipolaris sacchari* is phylogenetically closely related to *B. salkadehensis* (Figure 3.11). It differs by 4 base pairs (out of 456 bp) from *B. salkadehensis* in the ITS region. The GAPDH gene region of *Bipolaris salkadehensis* is not available for comparison. A sexual morph has not been described for this species.

29. *Bipolaris saccharicola* Y. Marín & Crous, Studies in Mycology 86: 101. [231].

Type material: Unknown country, unknown substratum, 1926, H. Atherton; holotype: CBS H-23114; ex-type culture: CBS 155.26 = MUCL 9693; Unknown country, from *Saccharum officinarum*, unknown date, R. R. Nelson, CBS 324.64; CBS 325.64 = DSM 62597 = MUCL 18220 = MUCL 9694 = NRRL 5241.

Known host: *Saccharum officinarum* [231].

Known distribution: Unknown country [231].

Bipolaris saccharicola and *B. maydis* have been found on *Saccharum officinarum.* It differs from *B. maydis* by the absence of a sexual morph, longer conidiophores and verruculose, more prominently curved conidia [231]. *Bipolaris cynodontis, B. sacchari, B. setariae* and *B. yamadae* have also been found on *Saccharum officinarum* [172], but only *B. saccharicola* is morphologically similar to *B. sacchari.* It differs by its longer and non-geniculate conidiophores (up to 300 µm *vs.* up to 900 µm tall in *B. saccharicola*) along with wider and more septate conidia (up to 9 distoseptate *vs.* up to 11 distoseptate in *B. saccharicola*). It is phylogenetically closely related to *B. brachiariae* and *B. maydis* (Figure 3.11). It differs by 7 base pairs (out of 490 bp) from *Bipolaris brachiariae* and by 7 base pairs (out of 496 bp) from *B. maydis* in the GAPDH gene region. *Bipolaris saccharicola* differs by 14 base pairs (out of 475 bp) from *B. brachiariae* and by 14 base pairs (out of 470 bp) from *B. maydis* in the ITS region.

30. *Bipolaris salkadehensis* Ahmadpour & Heidarian, Mycotaxon 120: 302. [242].

Type material: Iran, West Azerbaijan, Khoy City, Salkadeh village, on infected leaves of *Sparganium erectum*, 20 Sep. 2010, A. Ahmadpour; holotype: Bi-1 (includes ex-type culture), TUPP1366.

Known hosts: *Cladium mariscus* (Cyperaceae), *Sparganium erectum* (Typhaceae) [242].

Known distribution: Iran [242].

Bipolaris salkadehensis has overlapping conidial dimensions to *B. cynodontis* (50–70 × 10–15 μm *vs*. 40–80 × 12–18 μm) [171, 242]. *Bipolaris cynodontis* differs by its smaller conidia, lack of thick dark septa and with only bipolar germination [171]. *Bipolaris salkadehensis* is morphologically similar to *B. setariae* but *B. setariae* differs by having paler conidia with end cells demarcated by unthicken hyaline septa. *Bipolaris salkadehensis* is phylogenetically closely related to *B. sacchari* (Figure 3.11). It differs by 4 base pairs (out of 456 bp) from *B. sacchari* in the ITS region. The GAPDH gene region of *Bipolaris salkadehensis* is not available for comparison. A sexual morph has not been described for this species.

31. *Bipolaris salviniae* (J. J. Muchovej) Alcorn, Mycotaxon 41: 331. [238].

Type material: Australia, on Sach's agar + *Melinis minutiflora*, Nov. 1978, J.L. Alcorn; holotype: BRIP 12764a (*Cochliobolus melinidis*); Queensland, Maleny, on *M. minutiflora*, 24 May 1979, J. L. Alcorn; holotype: BRIP 12898 (*Bipolaris melinidis*); 1978, J.J. Muchovej, lectotype: BRIP 16571; lectotype culture: IMI 228224.

Known hosts: *Melinis minutiflora, Salvinia auriculata, Triticum aestivum*; (*Panicum maximum* var. *trichoglume, Setaria anceps*) [233].

Known distribution: Australia, Brazil, Paraguay [173]; (India) (Farr and Rossman 2020).

Sivanesan [171] proposed *Bipolaris melinidis* to be synonymized under *Drechslera curvispora* which was also accepted by Alcorn (1991). Alcorn (1991) and Manamgoda et al. [172] found that *Bipolaris curvispora* and *B. melinidis* are conspecific with *B. salviniae* based on morphological and phylogenetic analysis. There is no holotype specimen for this species as it was destroyed by insects and an isotype was not preserved. Alcorn (1991) designated IMI 228224 as the lectotype of *Drechslera salviniae* with BRIP 16571 as the isolectotype. This species forms a distinct lineage to other species (Figure 3.11).

32. *Bipolaris secalis* Sisterna Pl. Pathol. 38: 98. 1989.

Type material: Argentina, Buenos Aires, Los Hornos, from seed of *Secale cereale*, Aug. 1984, M.N. Sisterna; lectotype: IMI 286591; isolectotype: BRIP 14453 (ex-isotype culture included).

Known hosts: *Heliocarpus americanus* [217]; *Secale cereale* (Poaceae) [173]; (*Vitis vinifera*) [233].

Known distribution: Argentina [173]; Brazil [217]; Italy [233].

Sisterna [243] listed two specimens in different herbaria in the first publication of this species. Tan et al. [173] confirmed these two specimens as duplicates of the same type specimen and one of the specimens was designated the lectotype. *Bipolaris secalis* is phylogenetically closely related to *B. shoemakeri* (Figure 3.11). *Bipolaris shoemakeri* differs by having long, straight to flexuous conidiophores compared to the shorter (up to 300 μm) and apically geniculate conidiophores of *B. secalis*. This species differs from *B. shoemakeri* by 2 base pairs (out of 495 bp) in the GAPDH gene region and by 2 base pairs (out of 401 bp) in the ITS region.

33. *Bipolaris setariae* (Sawada) Shoemaker, Canad. J. Bot. 37: 884. [165].

Type material: Danish Fungi (Copenhagen): 527 (*Helminthosporium setariae* Lind.); Iconotype: MBT197972 (Lind 1913).

Known hosts: *Hevea brasiliensis* [220]; *Imperata cylindrica* [231]; *Pennisetum glaucum* (Poaceae), *Setaria imberbis, S. italica*, *S. lutescens*, *S. macrostachya* [172]; (*Agrostis tenuis, Antirrhinum majus*, *Avena sativa, Brachiaria mutica, B. reptans, Calathea* sp., *Caryota mitis, Chamaedorea elegans, C. seifrizii, Chrysalidocarpus lutescens*, *Cynodon* sp., *Dendrobium* sp., *Desmostachya bipinnata, Dianthus caryophyllus*, *Digitaria granularis, Echinochloa colonum, Echinochloa* sp., *Eleusine coracana, Eragrostis* sp., *Hordeum* sp., *Hordeum vulgare, Ischaemum rugosum, Manihot esculenta, Maranta arundinacea*, *M. leuconeura*, *Oryza sativa, Oryzopsis holciformis, Panicum clandestinum, P. fasciculatum, P. maximum, P. miliaceum, Paspalidium flavidum, Paspalum distichum, Pennisetum americanum, Persea americana, Rosa* sp., *Saccharum officinarum, Setaria faberi, S. geniculata, S. glauca, S. tomentosa, Sorghum* sp., *Triticum* sp., *Zea mays*) [233].

Known distribution: China [220]; Thailand [231]; USA [172]; (Australia, Canada, Egypt, Ethiopia, India, Korea, Myanmar, New Zealand, Pakistan, Peru, Sierra Leone, Taiwan, Turkey, Uganda, Venezuela) [233].

The type specimen of *Helminthosporium setariae* (Sawada) Shoemaker could not be found [172] and there are no illustrations of the type specimen in the protologue. The sexual morph of *Helminthosporium setariae* is *Ophiobolus setariae,* but its type specimen could not be located. Morphological characters of this species such as hilum morphology, septation and conidial shape fits the generic concept of *Bipolaris* [172]. *Bipolaris setariae* is phylogenetically closely related to *B. yamadae* (Figure 3.11). It differs from *B. yamadae* by 2 base pairs (out of 490 bp) in the GAPDH gene region and by 8 base pairs (out of 473 bp) in the ITS region.

34. *Bipolaris shoemakeri* Y. P. Tan & R. G. Shivas, Mycological Progress 15: 1206. [173].

Type material: Australia, Queensland, Mount Molloy, from leaf spot on *Ischaemum rugosum* var. *segetum* (Trin.) Hack., culture formed in vitro by crossing single-spored isolates, June 1987, J. L. Alcorn; holotype: BRIP 15929 (includes ex-type culture).

Known host: *Ischaemum rugosum* var. *segetum* [173].

Known distribution: Australia [173].

This species has been found on the host *Ischaemum rugosum* var. *segetum* which is commonly found in northern coastal area of Australia [244]. *Bipolaris cynodontis*, *B. oryzae* and *B. setariae* have also been recorded on *I. rugosum* [171–172]. *Bipolaris shoemakeri* differ by having longer conidiophores (up to 1.8 mm) compared to *B. cynodontis* (up to 170 µm), *B. oryzae* (up to 600 µm) and *B. setariae* (200 µm) [173]. *Bipolaris shoemakeri* is phylogenetically closely related to *B. secalis* (Figure 3.11). It differs by having long, straight to flexuous conidiophores compared to the shorter (up to 300 µm) and apically geniculate conidiophores of *B. secalis*. This species differs from *B. secalis* by 2 base pairs (out of 495 bp) in the GAPDH gene region and by 2 base pairs (out of 401 bp) in the ITS region.

35. *Bipolaris simmondsii* Y. P. Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Peregian Beach, on leaf spot on *Zoysia macrantha* Desv., 14 Nov. 1976, J. L. Alcorn; holotype: BRIP 12030 (includes ex-type culture).

Known host: *Zoysia macrantha* [173].

Known distribution: Australia [173].

Bipolaris simmondsii is only known from the type specimen on *Zoysia macrantha*. *Bipolaris simmondsii* is phylogenetically closely related to *B. heveae* and *B. microlaenae* (Figure 3.11). *Bipolaris heveae* differs by having conidia with a slightly protuberant hilum (3–4 µm) whereas *B. simmondsii* has an inconspicuous hilum [173]. This species differs by 13 base pairs (out of 490 bp) from *B. heveae* and by 10 base pairs (out of 490 bp) from *B. microlaenae* in the GAPDH gene region. It differs by 3 base pairs (out of 475 bp) from *B. heveae* and by 3 base pairs (out of 475 bp) from *B. microlaenae* in the ITS region.

36. *Bipolaris sivanesaniana* Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Atherton, from *Paspalidium distans* (Trin.) Hughes, 1 May 1987, J. L. Alcorn; holotype: BRIP 15847 (includes ex-type culture).

Known hosts: *Paspalidium distans, Setaria sphacelate* [173].

Known distribution: Australia [173].

Bipolaris sivanesaniana has been isolated from *Paspalidium distans* and *Setaria sphacelata* which possibly suggests a co-evolutionary relationship as these hosts are closely related [245]. This is the only *Bipolaris* species described on *Paspalidium distans*. *Bipolaris setariae*, has been isolated from *Paspalidium flavidum* but *B. sivanesaniana* differs by its longer conidiophores (up to 600 µm) compared to *B. setariae* (up to 200 µm long). *Bipolaris bicolor, B. cynodontis, B. maydis, B. oryzae, B. panici-milacei, B. sacchari, B. salviniae, B. setariae, B. sorokiniana, B. victoriae, B. yamadae* and *B. zeicola* have been recorded on the host *Setaria* [171,172]. *Bipolaris sivanesaniana* is phylogenetically closely related to *B. oryzae* and *B. panici-milacei* (Figure 3.11). It differs by its shorter conidia (60–86 µm *vs.* 63–

153 µm in *B. oryzae*) with fewer septa compared to *B. oryzae* (up to 8 *vs.* 14 in *B. oryzae*) [173]. *Bipolaris sivanesaniana* differs by its longer conidiophores compared to *B. panici-milacei* (up to 600 µm *vs.* up to 255 µm long in *B. panici-milacei*). This species differs by 3 base pairs (out of 486 bp) from *B. panici-milacei* in the GAPDH gene region. There is no base pair difference in the GAPDH gene between *Bipolaris sivanesaniana* and *B. oryzae.* It differs by 1 base pair (out of 473 bp) from *B. panici-milacei* and by 3 base pairs (out of 473 bp) from *B. oryzae* in the ITS region.

37. *Bipolaris sorokiniana* (Sorokin) Shoemaker, Canad. J. Bot. 37: 884. [165].

Type material: Russia, Sorokin. (1890), Proc. Biol. Soc. Imp. Univ. Kazan 22: 21–32; iconotype: MBT197973 (*Helminthosporium sorokinianum*). South Africa, Bloemfontein, on *Tribulus terrestris*, W. J. Jooste; holotype: PREM 44794 (*Drechslera multiformis*); ex-holotype culture: CBS 480.74.

Known hosts: *Amaranthus viridis* [222]; *Hordeum* sp.*, Hordeum vulgare, Lolium perenne, Phalaris arundinaceae, Secale cereal, Tribulus terrestris, Triticum aestivum* [172]; (*Aegilops cylindrica, Agropyron buonapartis, A. ciliare, A. cristatum, A. distichum, A. repens, A. trachycaulum* var. *trachycaulum, A. trachycaulum* var. *unilaterale, Agrostis capillaries, A. gigantea, A. palustris, Agrostis* sp., *A. stolonifera* var. *palustris, Allium* sp., *Alopecurus pratensis, Aneurolepidium chinense, Arrhenatherum elatius, Avena byzantina, A. sativa, Brachiaria plantaginea, Bromus inermis, B. japonicus, B. marginatus, B. uniloides, B. willdenowii, Broussonetia papyrifera, Buchloe dactyloides, Calluna vulgaris*, *Chloris virgata, Cicer arietinum, Cynodon dactylon, C. transvaalensis, Dactylis glomerata, Dendrobium* sp., *Digitaria sanguinalis, Echinochloa crus-galli, Ehrharta calycina, Eleusine coracana, E. indica, Elymus breviaristatus, E. canadensis, E. riparius, E. sibiricus, E. trachycaulus, E. virginicus, Elytrigia intermedia, E. repens, Eragrostis cilianensis, Fagopyrum esculentum, Festuca arundinacea, F. ovina, F. pratensis, F. rubra, Glycine max, Helianthus annuus, Holcus lanatus, Hordeum brevisubulatum, H. jubatum, H. leporinum, H. murinum, H. sativum, Hystrix patula, Lablab purpureus, Leymus angustus, L. cinereus, Lolium multiflorum, Linum usitatissimum, Lythrum salicaria, Medicago sativa, Microlaena stipoides, Microstegium vimineum, Miscanthus sinensis* var. *zebrinus, Oryza sativa, Panicum dichotomiflorum, P. lacromanianum, P.*

virgatum, Paspalum notatum, Pennisetum clandestinum, Phalaris arundinacea, P. canariensis, Phaseolus vulgaris, *Phleum pratense, Phleum* sp.*, Poa annua, P. pratensis, P. sylvestris, P. trivialis, Psathyrostachys juncea, Roegneria hirsuta, Saccharum* sp., *Secale montanum, Setaria viridis, Sporobolus vaginiflorus, Stenotaphrum secundatum, Taraxacum kok-saghyz*, *Tribulus terrestris, T. vulgare, Triticum durum, T. secale, Triticum* sp., *T. sphaerococcum, Zea mays, Zizania aquatica, Z. palustris*) [233].

Known distribution: Canada, India, Italy, Japan, New Zealand, South Africa, USA [172]; China [222]; (Australia, Bhutan, Brazil, Cameroon, Costa Rica, Cyprus, Denmark, Egypt, Ethiopia, Israel, Nicaragua, Nigeria, Poland, UK, Yugoslavia, Zimbabwe) [233].

Bipolaris sorokiniana can exhibit considerable morphological, physiological and genetic variation. It can infect a wide range of hosts including small grain cereals and a wide range of grasses, but oats are less susceptible to infection. This species produces the mycotoxins prehelminthosporal and sorokinianin. Manamgoda et al. [172] synonymized *Bipolaris multiformis* under *B. sorokiniana* based on morphological and phylogenetic analyses. *Bipolaris sorokiniana* is phylogenetically closely related to *B. crotonis* (Figure 3.11). It differs by 14 base pairs (out of 490 bp) from *B. crotonis* in the GAPDH gene region. It differs by 2 base pairs (out of 463 bp) from *B. crotonis* in the ITS region.

38. *Bipolaris subramanianii* Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Maclean Bridge, from leaf spot on *Setaria sphacelata*, 17 Mar. 1988, J. L. Alcorn; holotype: BRIP 16226 (includes ex-type culture).

Known host: *Setaria sphacelate* [173].

Known distribution: Australia [173].

Bipolaris subramanianii is only known from the type specimen. *Bipolaris cynodontis, B. maydis* and *B. zeicola* have also been recorded on the host *Setaria sphaecelata*. *Bipolaris subramanianii* differ by its longer conidiophores (up to 830 µm) compared to *B. cynodontis* (up to 170 µm) and *B. zeicola* (up to 250 µm) [173]. *Bipolaris subramanianii* differs by its longer conidia (70–130 µm) compared to *B.*

cynodontis (30–75 µm). *Bipolaris subramanianii* has straight to subcylindrical conidia compared to the slightly curved conidia that are broadest in the middle and taper towards the rounded ends of *B. cynodontis* and *B. zeicola* [173]. *Bipolaris maydis* differ by its distinctly curved conidia. *Bipolaris subramanianii* is phylogenetically closely related to *B. shoemakeri* and *B. secalis* (Figure 3.11). *Bipolaris subramanianii* has shorter conidiophores compared to *B. shoemakeri* (up to 1.8 mm), but longer conidiophores than *B. secalis* (up to 300 µm). *Bipolaris subramanianii* differ by its straight, slightly longer and thinner conidia compared to the slightly curved conidia of *B. shoemakeri* (70–80 × 13.5–15 µm). It differs by 6 base pairs (out of 495 bp) from *B. shoemakeri* and by 6 base pairs (out of 496 bp) *B. secalis* in the GAPDH gene region. This species differs by 10 base pairs (out of 473 bp) from *B. shoemakeri* and by 7 base pairs (out of 400 bp) *B. secalis* in the ITS region.

39. *Bipolaris urochloae* (K. M. Putterill) Shoemaker, Canad. J. Bot. 37: 885. [165].

Type material: UK, Baberton, on *Urochloa panicoides*, Mar. 1932, V.A. Wager 26148; holotype: IMI 38028.

Known hosts: *Urochloa panicoides* [172]; (*Dendrobium* sp., *Melinis minutiflora, Panicum laevifolium, P. maximum, Pennisetum americanum, P. glaucum, P. typhoides, Triticum aestivum, Urochloa helopus, U. mosambicensis, U. trichopus, Zea* sp.) [233].

Known distribution: Australia, UK [172]; (Brazil, Ethiopia, India, Pakistan, South Africa, USA, Zimbabwe) [233].

Bipolaris urochloae is associated with brownish black irregular lesions on *Urochloa panicoides* [171]. This species is characterised by large conidia with 8 up to 16 septa. This species forms a distinct lineage to other species (Figure 3.11).

40. *Bipolaris variabilis* Y. Marín, Y. P. Tan & Crous, Studies in Mycology 86: 104. [231].

Type material: Argentina, from leaf spots on *Pennisetum clandestinum*, 28 July 1986, col. M. N. Sisterna, isolated J. L. Alcorn; holotype: CBS H-23115; ex-type culture: CBS 127716 = BRIP 15349.

Known host: *Pennisetum clandestinum* [231].

Known distribution: Argentina [231].

This species has only been associated with brown to reddish leaf spots on *Pennisetum clandestinum*. *Bipolaris bicolor, B. cynodontis, B. maydis, B. sacchari, B. setariae, B. sorokiniana, B. urochloae* and *B. zeae* have been found on *Pennisetum* sp. [172]. *Bipolaris variabilis* can be easily identified based on its variable conidial size, shape and septation. It is phylogenetically closely related to *B. zeae* (Figure 3.11). *Bipolaris zeae* differ by its shorter conidiophores (up to 370 μm tall), and less septate conidia that are less variable in shape compared to *B. variabilis*. It differs from *B. zeae* by 1 base pair (out of 496 bp) in the GAPDH gene region. However, there is no base pair difference in the ITS region between *B. variabilis* and *B. zeae.*

41. *Bipolaris victoriae* (F. Meehan & H. C. Murphy) Shoemaker, Canad. J. Bot. 37: 882. [165].

Type material: USA, Iowa, Ames, Iowa Agricultural Experiment, on *Avena sativa* (cv. Boone), 25 Jul. 1946, M. Frances; holotype: BPI 431485; ex-type culture: CBS 327.64.

Known hosts: *Avena sativa* [172]; *Panicum virgatum* [226]; (*Agropyron cristatum, Agropyron* sp., *Chenopodium* sp., *Commelina benghalensis, Cymbopogon flexuosus, Dactylis glomerata, Digitaria ciliaris, Eleusine* sp., *Hordeum vulgare, Oryza sativa, Panicum maximum, Paspalum notatum, P. scrobiculatum, Phalaris arundinacea, Phleum pretense, Setaria* sp., *Setaria sphacelata, S. verticillata, S. viridis, Sorghum vulgare, Triticum* sp., *Zea mays*) [233].

Known distribution: Canada, USA [172, 226]; (Australia, Brazil, India, Kenya, UK, Zambia, Zimbabwe) [233].

An epitype is designated for this species from culture collected from the original host and location [172]. Meehan and Murphy [246] first reported *Helminthosporium victoriae* as the cause of "Victoria blight" on oats which is synonymised under *Bipolaris victoriae*. This species is phylogenetically closely related to *B. microstegii* and *B. zeicola* (Figure 3.11). *Bipolaris victoriae* differs from *B. zeicola* as it contains only the MAT-2 (mating type) locus, whereas *B. zeicola* contain either one or both MAT-1 and MAT-2. Mating type genes are vital for genotypic variation that allow adaptation to environmental changes. This species differs by 1 base pair (out of 490 bp) from *Bipolaris microstegii* and *B. zeicola* in the

GAPDH gene region. It differs by 2 base pairs (out of 469 bp) from *Bipolaris microstegii* and by 5 base pairs (out of 464 bp) from *B. zeicola* in the ITS region.

42. *Bipolaris woodii* Tan & R. G. Shivas, Mycological Progress 15: 1206 [173].

Type material: Australia, Queensland, Goondiwindi, from *Paspalidium caespitosum* C. E. Hubb., 25 Apr. 1977, J. Brouwer; holotype: BRIP 12239 (includes ex-type culture).

Known host: *Paspalidium caespitosum* [173].

Known distribution: Australia [173].

Bipolaris woodii is only known from the type specimen on *Paspalidium caespitosum*. *Bipolaris setariae* and *B. sivanesaniana* have been recorded on *Paspalidium flavidum* and *P. distans* respectively [173]. This species differs by its shorter conidiophores (up to 250 µm) compared to *B. sivanesaniana* (up to 600 µm) [173]. It is phylogenetically closely related to *B. microstegii, B. victoriae* and *B. zeicola* (Figure 3.11). *Bipolaris woodii* differs by its shorter conidiophores compared to *B. microstegii* (up to 750 µm) whereas *B. woodii* differ by its smaller and darker conidia compared to *B. victoriae* (69–76 × 12.5–13.5 µm *vs.* 40–120 × 12–19 µm in *B. victoriae*) [171]. This species differs by its darkened and conspicuous hilum compared to the inconspicuous hilum of *B. zeicola*. It differs by 6 base pairs (out of 497 bp) from *B. microstegii,* 7 base pairs (out of 491 bp) from *B. victoriae* and by 7 base pairs (out of 491 bp) from *B. zeicola* in the GAPDH gene region. This species differs by 10 base pairs (out of 473 bp) from *B. microstegii,* 11 base pairs (out of 474 bp) from *B. victoriae* and by 14 base pairs (out of 469 bp) from *B. zeicola* in the ITS region.

43. *Bipolaris yamadae* (Y. Nisik.) Shoemaker, Canad. J. Bot. 37: 884. [165].

Type material: Japan, on *Panicum miliaceum*, Y. Nisikado (1928a), Rept. Ohara. Inst. Agr. Research 4: Plate XIII fig. 1 J. A. Stevenson Mycology Library, USDA-ARS, Beltsville, Maryland, USA; (*Helminthosporium yamadae*) iconotype "MBT197976"; on *Panicum miliaceum*, deposited Nov. 1929, Y. Nisikado No. 273; epitype: CBS H-7221.

Known hosts: *Panicum capillare* [172]; *P. miliaceum* [173]; (*Oryza* sp*., Panicum implicatum, P. maximum, Saccharum* sp., *Setaria plicata*) [233].

Known distribution: Japan [173]; USA [172]; (China, Cuba, India) [233].

Manamgoda et al. [172] designated the specimen CBS H-7221 as an epitype of *Helminthosporium yamadai* (*Bipolaris yamadae*) with an ex-epitype culture. *Bipolaris yamadae* is phylogenetically closely related to *B. setariae* and *B. microconidica*. This species forms a distinct lineage to other species (Figure 3.11). A sexual morph is not recorded in association with this species.

44. *Bipolaris zeae* Sivan., Trans. Brit. Mycol. Soc. 84: [172].

Type material: Australia, on *Zea mays*, 19 Jan. 1975, P. E. Mayers 20425b; holotype: IMI 202085.

Known hosts: *Helianthus tuberosus* [227]; *Panicum virgatum, Sorghum bicolor, Triticum aestivum* [172]; *Zea mays* [173]; (*Acer truncatum, Alloteropsis semialata, Brachiaria decumbens, Cenchrus ciliaris, Cynodon dactylon, Dactylis* sp., *Imperata cylindrica* var. *major, Paspalum* sp., *Pennisetum americanum*, *P. clandestinum, P. glaucum*, *P. typhoides, Sorghum halepense*, *Triticum vulgare*) [233].

Known distribution: Australia [173]; Canada, Japan, USA [172]; China [227]; (Argentina, Brazil, Colombia, India, Iran, Taiwan) [233].

Bipolaris zeae can be found on a wide range of hosts and it is not regarded as a serious pathogen [172]. This species is phylogenetically closely related to *B, variabilis* (Figure 3.11). *Bipolaris zeae* is characterised by shorter conidiophores (up to 370 μm tall), and less septate conidia that are less variable in shape compared to *B. variabilis* [172]. It differs from *B. variabilis* by 1 base pair (out of 496 bp) in the GAPDH gene region. However, there is no base pair difference in the ITS region between *B. variabilis* and *B. zeae.* The sexual morph was obtained by pairing *Bipolaris zeae* isolates from *Pennisetum clandestinum* in Taiwan.

45. *Bipolaris zeicola* (G. L. Stout) Shoemaker, Canad. J. Bot. 37: 885. [165].

Type material: USA, Illinois, Dixon Lee Co., on *Zea mays*, 26 Sep. 1926, Nat. Hist. Surv. Acc. No 19884 (*Bipolaris zeicola*), holotype; Ohio, on leaf of *Z. mays*, R. Hite; epitype: BPI 892947; holotype: BPI 626376.

Known hosts: *Arundo donax, Bouteloua curtipendula, Eragrostis cilianensis, Sorghum* sp. [172]; *Zea mays* [173]; (*Brachiaria foliosa, Chloris gayana, C. verticillata,* Coffea arabica, *Cynodon dactylon*) [233].

Known distribution: Japan, USA [172]; (Australia, Brazil, Canada, China, Denmark, Egypt, New Zealand, Zimbabwe) [233].

Bipolaris zeicola causes oval to circular or sometimes irregular, straw-coloured or chocolate brown spots on *Zea mays*. Three pathogenic races of this species have been identified based on symptoms on maize [172]. Race one produces HC toxin which causes severe damage on maize leaves. Race two does not produce HC toxins. Race 3 also causes severe damage on rice and maize, but it produces the toxin, BZR-toxin which is responsible for leaf chlorosis on rice. *Bipolaris zeicola* is phylogenetically closely related to *B. microstegii* and *B. victoriae* (Figure 3.11). It differs from *B. zeicola* as it contains only the MAT-2 locus, whereas *B. zeicola* contain either one or both MAT-1 and MAT-2. This species differs by 1 base pair (out of 490 bp) from *Bipolaris victoriae* and *B. microstegii* in the GAPDH gene region. It differs by 2 base pairs (out of 469 bp) from *Bipolaris victoriae* and by 5 base pairs (out of 464 bp) from *B. microstegii* in the ITS region.

CHAPTER 4

INVESTIGATING SPECIES BOUNDARIES IN *COLLETOTRICHUM*[3]

Abstract

Colletotrichum is one of the most important plant pathogenic genera that is responsible for numerous diseases which can have a profound impact on the agricultural sector. Species delineation is difficult due to a lack of distinctive phenotypic variation. Therefore, in this study three different genomic approaches based on phylogenetic, evolutionary and coalescent-based methods are applied to establish robust species boundaries. The reliability of five different DNA barcodes was also assessed to provide further insights into species delineation. The ITS region can resolve the placement of taxa up to the species complex level. The *GAPDH* and *TUB2* markers are determined to be the most informative for most complexes. However, no single marker could discriminate between species in all complexes, therefore different molecular approaches based on multi-locus datasets is recommended. This is the first study to provide an estimated divergence time for all species complexes in *Colletotrichum.* The estimated divergence time for species complexes ranged between 4.8 to 32.2 MYA. Based on the high level of congruent results obtained from the different molecular approaches, a new species complex, the *Colletotrichum agaves* complex is introduced. This complex consists of five taxa which are characterised by the presence of straight or slightly curved conidia with obtuse apices. This complex consists of five taxa [1]

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I declare that my contribution to this chapter was 95%.

which are characterised by the presence of straight or slightly curved conidia with obtuse apices. This study shows that coalescent approaches and multi-locus phylogeny are crucial to establish species boundaries in *Colletotrichum*. The taxonomic placement of three singleton taxa *Colletotrichum axonopodi, C. cariniferi* and *C. parallelophorum* is revised. We accept 248 species and provide recommendations regarding species boundaries in the graminicola-caudatum complex.

Keywords: BEAST, General Mixed Yule Coalescent Method, Multi-rate Poisson Tree Process, *Sordariomycetes,* Taxonomy

4.1 Introduction

Colletotrichum was introduced by Corda [247] with *C. lineola* as the type species and it is the only member of *Glomerellaceae* (*Glomerellales*, *Sordariomycetes*) [20, 168]. *Colletotrichum* species are endophytes, pathogens and saprobes [248–251]. As endophytes, they are one of the most widely distributed genera and can produce a range of secondary metabolites [251]. As plant pathogens, species can cause anthracnose and postharvest fruit rots [252], leading to yield loss especially in high-value crops worldwide. Some species are associated with a single host while some can infect multiple hosts, which hinders effective disease management [253]. *Colletotrichum* species have also been known to cause human infections for example, *C. dematium, C. gloeosporioides* and *C. graminicola* can cause Keratitis [254–255].

Accurate species delineation is vital especially for pathogenic genera specifically in studies focusing on biodiversity, conservation and evolution as well as for the establishment of quarantine measures. Morphology based delimitation of species is problematic due to overlapping morphological characters in asexual morphs and the lack of sexual morphs in this genus [74]. The reliability of morphological characters is also affected due to variation in culture conditions such as the choice of synthetic media among different studies [76, 257–258]. The application of a standard medium such as potato dextrose agar (PDA) for morphological studies of *Colletotrichum* would minimize the phenotypic variability within isolates, thus potentially maximizing

differences between species [74]. Several species have been wrongly named based only on their host's specificity [74]. However, our understanding of host specificity in *Colletotrichum* is limited as most studies have mainly focused on economically important crops or ornamental crops [74]. Accurate molecular identification of species is also hindered due to the lack of ex-type or authenticated sequences [76].

There is also no agreement on the barcodes that should be used for species identification as different markers have been used in different studies. Up to 13 different markers have been used for species delineation in *Colletotrichum* which is impractical in terms of time and cost [60, 102, 256–257, 259]. Many of these markers provide little in resolution for species delineation as the phylogenetic significance of a marker varies among complexes [60]. Over 900 epithets are listed in Index Fungorum [30] under *Colletotrichum*, which comprises 247 accepted species [52]. The majority of the species are grouped into 14 species complexes namely acutatum, boninense, caudatum, dematium, destructivum, dracaenophilum, gigasporum, gloeosporioides, graminicola, magnum, orbiculare, orchidearum, spaethianum and truncatum [231, 249, 256]. Cai et al. [76] recommended a polyphasic approach including multi-loci sequence analyses, analyses of ecological, geographical and morphological data for better taxonomic resolution for this genus, however such polyphasic approaches can be difficult to apply. The identification of cryptic species based on the phylogenetic species concept can lead to an artificial increase in the number of taxa in a group. Therefore, advances in coalescent methods could represent a new approach for species delineation for cryptic species as they can provide insights in divergence and evolutionary relationships among species. Coalescent methods can be based on a maximum likelihood or Bayesian function whereby species delimitation models are evaluated differently [260]. In the maximum likelihood approach, point estimates are used for the genealogies at each locus and for the population. The Bayesian approach incorporates genealogical uncertainty by estimating gene trees directly from sequence data for each locus.

Coalescent based methods have been commonly used for species delineation in the plant and animal kingdom, but rarely in fungal research. Coalescent based methods such as the multi-rate Poisson tree processes (mPTP) and the general mixed Yule-coalescent method (GMYC) were designed for single-locus analysis, but have successfully been applied to concatenated multi-locus datasets [261]. These approaches

can incorporate the process of lineage sorting and the presence of incongruent genomic regions into phylogenetic estimation procedures [262]. Coalescent based method computes the likelihood of a species tree from the probabilities of the individual gene trees which can be used to investigate causes of gene trees discordance [102]. The species boundaries detected by coalescent based methods are considered as initial hypotheses and should therefore be validated using findings from for example the phylogenetic species concept. The GMYC method is based on the assumption that independent evolution leads to the appearance of distinct genetic clusters which are separated by longer internal branches [263]. It delimits these clusters by finding the maximum likelihood solution for a model that combines diversification between species (based on a Yule model) and genealogical branching within species. The advantage of the GMYC framework is that it allows for statistical inference and hypothesis testing across the entire sampled clade [264]. The advantage of the mPTP method is that it incorporates different levels of intraspecific genetic diversity derived from differences in the evolutionary history or sampling of each species [265]. We therefore aim to elucidate species and species complex boundaries within *Colletotrichum* by using phylogenetic analyses, evolutionary analyses coupled with coalescent-based methods including mPTP and GMYC as well as determine the phylogenetic significance of five different DNA barcodes for species delineation.

4.2 Materials and Methods

4.2.1 Taxon Sampling

The type species included in Jayawardena et al. [52] are used as the starting point for this study. The dataset includes the genes actin (*ACT*), β-tubulin2 (*TUB2*), chitin synthase (*CHS-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the internal transcribed spacers (ITS) region of all the species that have been published until June 2020. These DNA regions were selected based on their previously reported usefulness and wider availability of genomic data in GenBank.

4.2.2 Phylogenetic Analyses

Phylogenetic analyses were performed using seven datasets: (1) single genes (*ACT*, *CHS-1*, *GAPDH*, ITS, *TUB2*), (2) multi-locus dataset, (3) a larger dataset. The multi-locus dataset included only the type species and the larger dataset included up to two strains for each of the type species. The selected strains were from different geographic locations and different hosts. The sequences were aligned by MAFFT v. 7.036 (https://mafft.cbrc.jp/ alignment/server/) using default settings. The alignments were manually improved using BioEdit v. 7.0.5.2 [188] and AliView v. 1.26 [266].

Maximum likelihood, maximum parsimony and Bayesian inference methods were used for the phylogenetic reconstructions of the multi-locus and individual genes. Maximum likelihood analyses were performed by running 1000 pseudoreplicates using RAxML-HPC2 on XSEDE (8.2.8) [189] in the CIPRES Science Gateway platform [190]. Maximum parsimony analyses were conducted using PAUP v.4.0b [267] using the heuristic search option with 1000 bootstrap replicates. The Tree Length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were calculated. The best-fitting substitution models were determined by jModelTest version 2.1.10 [268]. Bayesian inference analyses were conducted using MrBayes v. 3.2.2 [193]. Six simultaneous Markov chains were run for 50,000,000 generations and trees were sampled every 1000th generation. The suitable burn-in phases were determined using Tracer version 1.7 [194] and were discarded. The remaining trees were used to calculate posterior probabilities in the majority rule consensus tree. FigTree v. 1.4.0 was used to view the resulting trees [269] and the final layout was done with Adobe Illustrator CS v. 22.1 (Adobe Systems, USA).

4.2.3 Divergence Time Estimation

Divergence times were estimated in BEAST 2.6.2 [270] based on the best fitting substitution models used for phylogenetic analyses. The dataset (ITS, *GAPDH*, *CHS-1*, *ACT* and *TUB2*) was partitioned and the XML file was prepared in BEAUTI 2.6.2. An uncorrelated relaxed clock model with lognormal distribution was used for the fossil analysis. A Yule speciation process birth rate was used for the tree prior. The fossil *Protocolletotrichum deccanensis* represents an ancient lineage of extant *Colletotrichum* and it was used to constrain the common ancestor of *Colletotrichum* to the minimum

age of 61 million years ago (MYA) [271–273]. The analysis was carried out for 200 million generations and trees were sampled every 10,000th generations. The output was visualized in Tracer version 1.7 to ensure an effective sample size (ESS) greater than 200 [274]. Five independent analyses were performed to ensure congruence. The first 10% of the generated trees representing the burn-in phase were discarded. The remaining trees were combined using LogCombiner 2.6.2, summarized in TreeAnnotator 2.6.2 (BEAST packages) and visualized with FigTree v.1.4.0.

4.2.4 Multi-Rate Poisson Tree Processes

The multi-rate Poisson tree processes (mPTP) incorporates different levels of intraspecific genetic divergences for each species [265]. The RAxML tree was used as the input file as this approach takes in a binary phylogenetic tree. The phylogenetic tree was rooted in mPTP v. 0.2.4 at *Monilochaetes camelliae* (BRIP 24607) and *Monilochaetes infuscans* (CBS 869.96) as the outgroup taxa.

4.2.5 The General Mixed Yule-Coalescent Method

The GMYC method uses a likelihood approach, which is a combination of the neutral coalescent theory with Yule speciation model [264]. It aims to detect shifts in branching rates between intra- and interspecific relationships by comparing two models, the null and GMYC models. Under the null model, all the individuals belong to a single species or population [264]. In the GMYC model, a Yule speciation and extinction model is applied whereas a coalescent process is applied to intraspecific relationships [275]. If the GMYC model fits the data significantly better than the null model, the threshold can be used to estimate the number of species. The GMYC approach takes in an ultrametric and bifurcating tree file. The input tree was prepared from the output tree from the Beast analysis with TreeAnnotator 2.6.2 using the maximum clade credibility method. The GMYC analyses were performed in R using the splits package v. 1.0-19 [276]. The analyses were performed using the single and multiple-threshold method. In the single threshold method, a single transition model is applied which assumes that all species have the same coalescent branching rate whereas in the multiple-threshold method variable transition is applied from coalescent to speciation across different clades [277]. All the analyses in this study (divergence time estimation, mPTP and GMYC) were repeated using the larger dataset.

4.3 Results

4.3.1 Phylogenetic Analyses

The ITS dataset consisted of 253 taxa with 319/635 conserved sites, 292/635 variable sites and 212/635 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree out of 1000 (CI = 0.375, RI = 0.882, RC = 0.331, HI = 0.625, Tree Length = 1260). The best scoring RAxML tree had a final likelihood value of -7658.697005. The general time reversible (GTR) model with a discrete gamma distribution plus invariant site (GTR+I+G) substitution model was implemented in the Bayesian analysis of the ITS dataset. The *GAPDH* dataset consisted of 220 taxa with 52/461 conserved sites, 382/461 variable sites and 317/461 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree out of 1000 (CI = 0.314, RI = 0.858, RC = 0.270, HI = 0.686, Tree Length = 2723). The best scoring RAxML tree had a final likelihood value of -12518.203342. The Hasegawa-Kishino-Yano (HKY) model with a discrete gamma distribution plus invariant site (HKY+I+G) substitution model was implemented in the Bayesian analysis of the *GAPDH* dataset. The *TUB2* dataset consisted of 238 taxa with 195/619 conserved sites, 365/619 variable sites and 307/619 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree out of 1000 (CI = 0.268, RI = 0.856, RC = 0.230, HI = 0.732, Tree Length = 2857). The best scoring RAxML tree had a final likelihood value of -14703.137100. A HKY+I+G model was implemented in the Bayesian analysis of the TUB2 dataset. The CHS-1 dataset consisted of 202 taxa with 155/282 conserved sites, 127/282 variable sites and 100/282 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree out of 1000 (CI = 0.228, RI = 0.813, RC = 0.186, HI = 0.772, Tree Length = 871). The best scoring RAxML tree had a final likelihood value of -4786.387059. A GTR+I+G model was implemented in the Bayesian analysis of the *CHS-1* dataset. The *ACT* dataset consisted of 235 taxa with 71/340 conserved sites, 249/340 variable sites and 206/340 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree out of 1000 (CI = 0.319, RI = 0.862, RC = 0.275, HI = 0.681, Tree Length = 1594). The best scoring RAxML tree

had a final likelihood value of -8269.293999. A GTR+I+G model was implemented in the Bayesian analysis of the *ACT* dataset.

The multi-locus dataset (ITS, *GAPDH*, *CHS-1*, *ACT* and *TUB2*) consisted of 253 taxa with 792/2337 conserved sites, 1415/2337 variable sites and 1142/2337 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree (Figure 4.1) out of 1000 (CI = 0.282, RI = 0.844, RC = 0.238, HI = 0.718, Tree Length = 9953). The best scoring RAxML tree had a final likelihood value of -52302.678794. With some exceptions, all three phylogenetic approaches resulted in similar tree topology and major clades based on the multi-locus dataset. The gigasporum and dracaenophilum complexes formed a sister clade to the gloeosporioides/truncatum and agaves/boninense complexes in the Bayesian Inference analysis (0.99 BYPP). In the maximum parsimony and maximum likelihood analysis, the gigasporum and dracaenophilum complexes are closely related to the orchidearum/magnum/orbiculare complexes (>70% BT). Two taxa from the graminicola complex (*C. endophytum* and *C. falcatum*) clustered together with strong support (1.00 BYPP), forming a basal clade to the caudatum complex in the Bayesian Inference analysis. The singleton *C. hsienjenchang* clustered with the singleton *C. metake* at the basal lineage of the graminicola complex in the maximum likelihood and Bayesian Inference analysis (68% MLBT/0.81 BYPP). In the maximum parsimony analysis, *C. metake* formed a sister taxon to the graminicola complex with low support (<50% BT) and *C. hsienjenchang* formed a sister taxon to the spaethianum complex with low support (<50% BT). The caudatum complex formed an inner clade in the graminicola complex based on all three phylogenetic analyses (98% MLBT/84% MPBT/1.00 BYPP). Five singleton taxa *C. agaves*, *C. euphorbiae*, *C. ledebouriae*, *C. neosansevieriae* and *C. sansevieriae* formed a new complex in the maximum parsimony, maximum likelihood and the Bayesian inference analyses with strong support (100% MLBT/100% MPBT/1.00 BYPP). These species formed a sister clade to the boninense complex in all the phylogenetic analyses. The singleton *C. parallelophorum* (MFLUCC 14-0083) formed a sister taxon to *C. coelogynes* in the dracaenophilum complex with strong support (100% MLBT/100% MPBT/1.00 BYPP). The singleton *C. cariniferi* (MFLUCC 14-0100) clustered in the dracaenophilum complex, forming a basal clade to *C. parallelophorum* and *C. coelogynes* (80%

MLBT/52% MPBT/0.99 BYPP). The singleton *C. axonopodi* (IMI279189) formed a sister taxon to *C. hanaui* in the graminicola complex with strong support (100% MLBT/63% MPBT/0.94 BYPP).

The phylogenetic trees from the single gene were compared to the phylogenetic tree from the multi-locus dataset in terms of topology and support for species relationships. The ITS dataset resolved the placement of all the taxa up to complex level, except *C. cereale* and *C. orchidis* which clustered in the spaethianum and destructivum complexes respectively. The caudatum complex formed an inner clade in the graminicola complex based on all three phylogenetic analyses of the ITS region (90% MLBT/83% MPBT/1.00 BYPP). The truncatum complex formed a sister clade to the boninense complex in the ITS dataset (95% MLBT/97% MPBT/1.00 BYPP) whereas the truncatum complex formed a sister clade to the gloeosporioides complex in the multi-locus dataset (100% MLBT/85% MPBT/1.00 BYPP). Based on the ITS dataset, the dracaenophilum complex is closely related to the gigasporum complex in the maximum parsimony and the Bayesian inference analyses (1.00 BYPP) whereas it forms a sister clade to the orchidearum/magnum complexes in the maximum likelihood analysis (56% MLBT). A new complex with five taxa *C. agaves*, *C. euphorbiae*, *C. ledebouriae*, *C. neosansevieriae* and *C. sansevieriae* was formed in the maximum parsimony, maximum likelihood and the Bayesian inference analyses based on the ITS dataset with strong support (100% MLBT/99% MPBT/1.00 BYPP). *Colletotrichum axonopodi* formed a sister taxon to *C. hanaui* in the graminicola complex based on the ITS dataset (99% MLBT/72% MPBT/0.97 BYPP). *Colletotrichum parallelophorum* formed a sister taxon to *C. cariniferi* in the dracaenophilum complex with strong support only in the maximum likelihood analysis based on the ITS dataset (93% MLBT). The single marker *GAPDH* and *TUB2* recovered the highest number of species in the acutatum, boninense, dematium, destructivum, dracaenophilum, magnum and spaethianum complexes as monophyletic with strong support (≥70% BT/≥0.90 BYPP). The *TUB2* gene was the most informative in the gloeosporioides, graminicola and orchidearum complexes whereas *GAPDH* was the most informative in the gigasporum, orbiculare and truncatum complexes. The *ACT* gene was the next best marker in the graminicola and spaethianum complexes. The *GAPDH*, *ACT* and *TUB2* markers were all equally effective in species recognition in the caudatum and agaves complex.

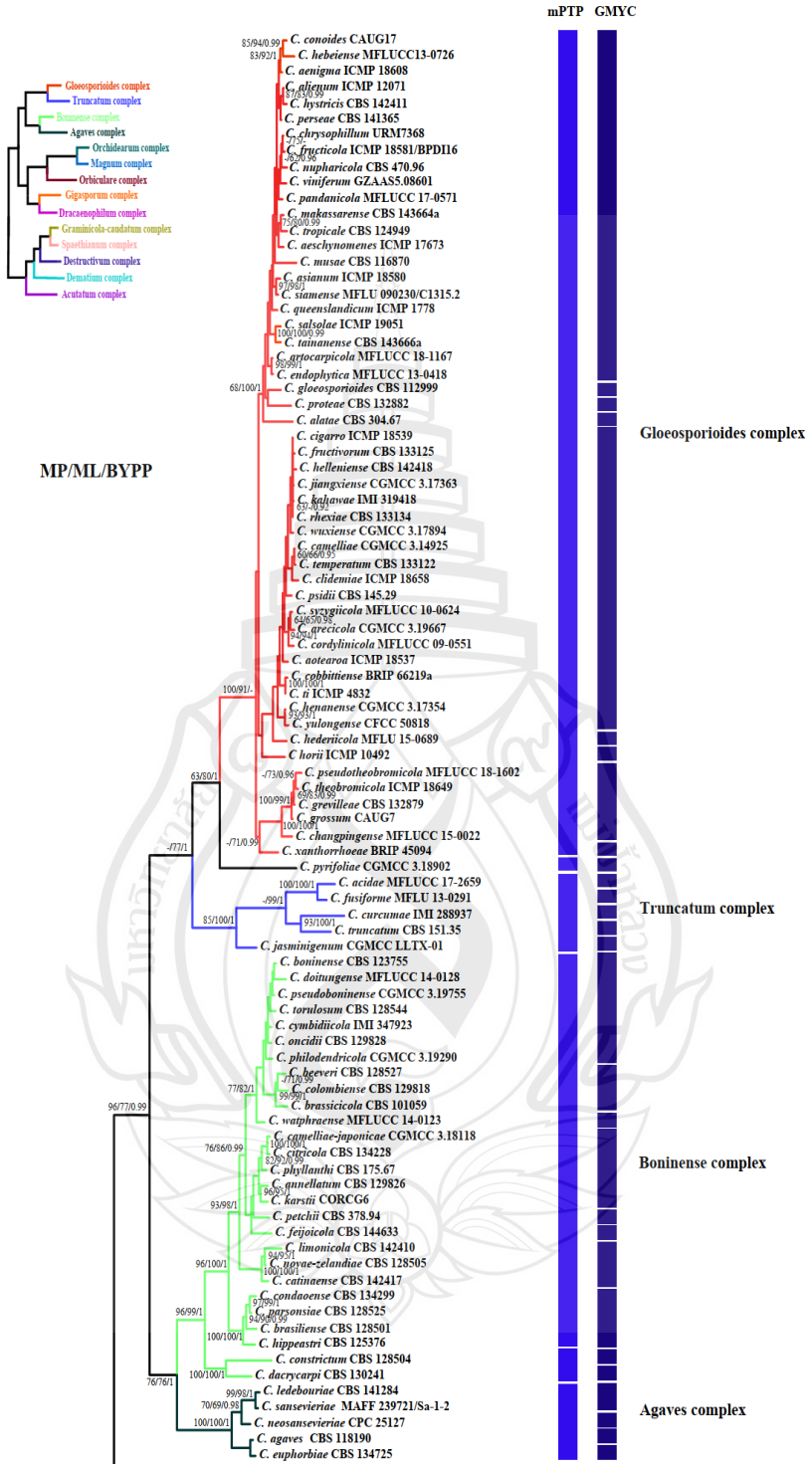


Figure 4.1 Phylogenetic analysis, mPTP and GMYC of *Colletotrichum* results

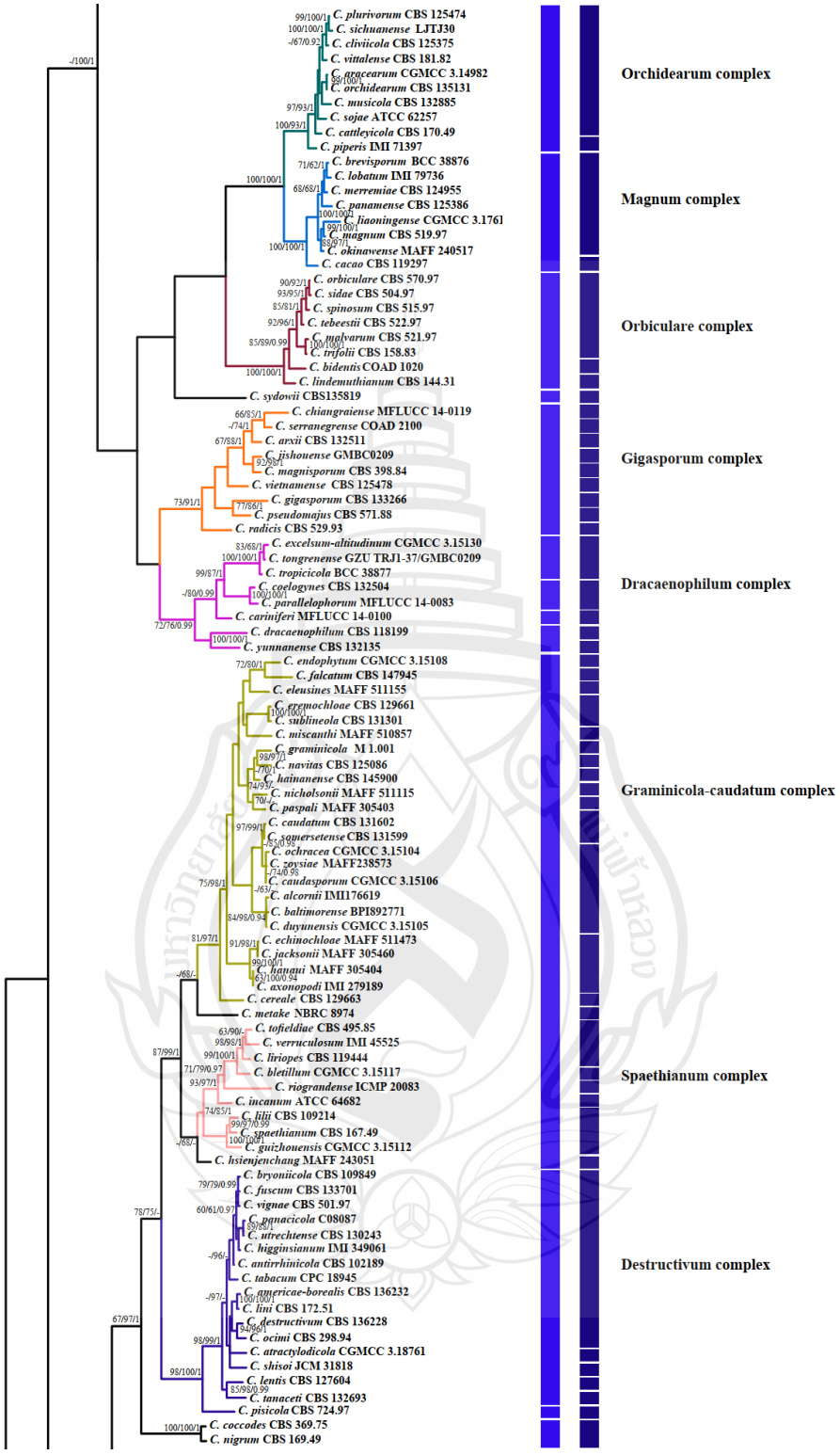
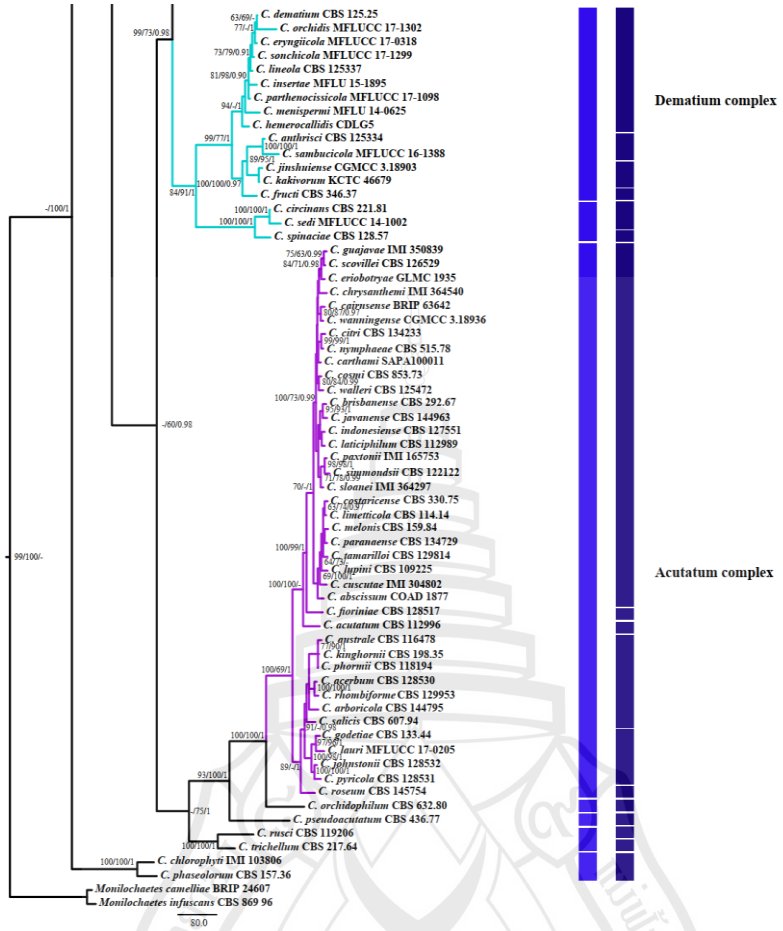


Figure 4.1 (continued)



Note One of the most parsimonious trees generated by maximum parsimony analysis of combined ITS, *GAPDH*, *CHS-1*, *ACT* and *TUB2* dataset of *Colletotrichum* type sequences. RAxML bootstrap support and maximum parsimony values ≥60% (BT) as well as Bayesian posterior probabilities ≥0.90 (BYPP) are shown respectively near the nodes. The ex-type strains are in bold and the scale bar indicates 80 changes. The tree is rooted with *Monilochaetes camelliae* (BRIP 24607) and *Monilochaetes infuscans* (CBS 869.96). The columns present the results of mPTP and GMYC based on the type sequences. The figure in the upper left corner represents the placement of all the complexes based on the maximum parsimony analysis. Each complex is represented by a different colour.

Figure 4.1 (continued)

4.3.2 Coalescent-based Species Delimitation Methods

The mPTP method recovered 26 entities in the multi-locus dataset (Figure 4.1). The acutatum, gigasporum, gloeosporioides, magnum, orbiculare, orchidearum and truncatum complexes were each recovered as distinct taxonomic entities. *Colletotrichum axonopodi*, *C. hsienjenchang, C. metake* as well as all the species from the caudatum, graminicola, and spaethianum complexes were recovered as conspecific. The remaining complexes were split into several entities (boninense into 2 entities; dematium 2; destructivum 2; dracaenophilum into 3 entities). *Colletotrichum sansevieriae*, *C. ledebouriae*, *C. neosansevieriae*, *C. euphorbiae* and *C. agaves* were all recovered as one taxonomic species. The singleton *C. parallelophorum* and *C. coelogynes* from the dracaenophilum complex were recovered as conspecific.

The GMYC approach detected 97 entities based on the multi-locus dataset in the single and multiple threshold method (Figure 4.1). The GMYC model provided a better fit to the ultra-metric tree based on the likelihoods for the null (L_0) and GMYC (L_{GMYC}) models (single threshold: L_0: 396.29, L_{GMYC}: 403.0176, likelihood ratio (LR): 13.4552; multiple threshold: L_0: 396.29, L_{GMYC}: 409.6819, LR: 26.78377). All the singletons were recovered as conspecific with few exceptions as detailed in Figure 1. *Colletotrichum axonopodi* and all the species from the graminicola complex were recovered as one taxonomic unit. *Colletotrichum parallelophorum* and all species from the dracaenophilum complex were recovered as conspecific. Both the magnum and orchidearum complexes were divided into two distinct entities. The remaining complexes were divided into several entities (orbiculare complex into 3 entities; caudatum 3; graminicola 12; spaethianum 5; dematium 6; boninense 10; gloeosporioides 9; truncatum 5; dracaenophilum 4; gigasporum 9; destructivum 6; acutatum into 6 entities). *Colletotrichum sansevieriae* and *C. ledebouriae* were recovered as conspecific.

4.3.3 Divergence Time Estimation

The topology of the maximum clade credibility (MCC) tree from the BEAST analysis (Figure 4.2) was generally congruent with the result from the maximum likelihood, maximum parsimony and Bayesian inference analysis with some exceptions. The gigasporum and dracaenophilum complexes formed a sister clade to

the agaves/boninense and gloeosporioides/truncatum complexes similar to the Bayesian Inference analysis (0.98 BYPP). Three species from the graminicola complex *C. endophytum*, *C. falcatum* and *C. eleusines* formed a basal clade to the caudatum complex. The caudatum complex formed an inner clade in the graminicola complex. *Colletotrichum axonopodi* formed a sister taxon to *C. hanaui* in the graminicola complex with strong posterior probability (0.99 BYPP). *Colletotrichum parallelophorum* formed a sister taxon to *C. coelogynes* in the dracaenophilum complex with strong posterior probability (1.00 BYPP). *Colletotrichum cariniferi* clustered in the dracaenophilum complex, forming a basal clade to *C. parallelophorum* and *C. coelogynes* (0.93 BYPP).

The crown age of the caudatum complex was estimated at 9.5 MYA, spaethianum complex at 15.3 MYA, destructivum complex at 13.9 MYA, acutatum complex at 9 MYA, dematium complex at 32.2 MYA, gloeosporioides complex at 9.9 MYA, truncatum complex at 22.9 MYA, boninense complex at 19.2 MYA, gigasporum complex at 21.1 MYA, dracaenophilum complex at 21 MYA, orchidearum complex at 4.8 MYA, magnum complex at 6.2 MYA and the orbiculare complex at 6.9 MYA (Table 4.1). *Colletotrichum sansevieriae*, *C. ledebouriae*, *C. neosansevieriae*, *C. euphorbiae* and *C. agaves* formed a separate complex with a crown age of 9.1 MYA. The stem age of this complex and the boninense complex was estimated at 33.3 MYA. The caudatum and graminicola complex (including the singleton *C. axonopodi*) diverged at 18.6 MYA. The stem age of the gigasporum and dracaenophilum complex (including the singleton *C. parallelophorum* and *C. cariniferi*) was estimated at 39.1 MYA. The orchidearum and magnum complex diverged at 14.8 MYA. The orbiculare complex diverged from the orchidearum and magnum complex at 55.9 MYA.

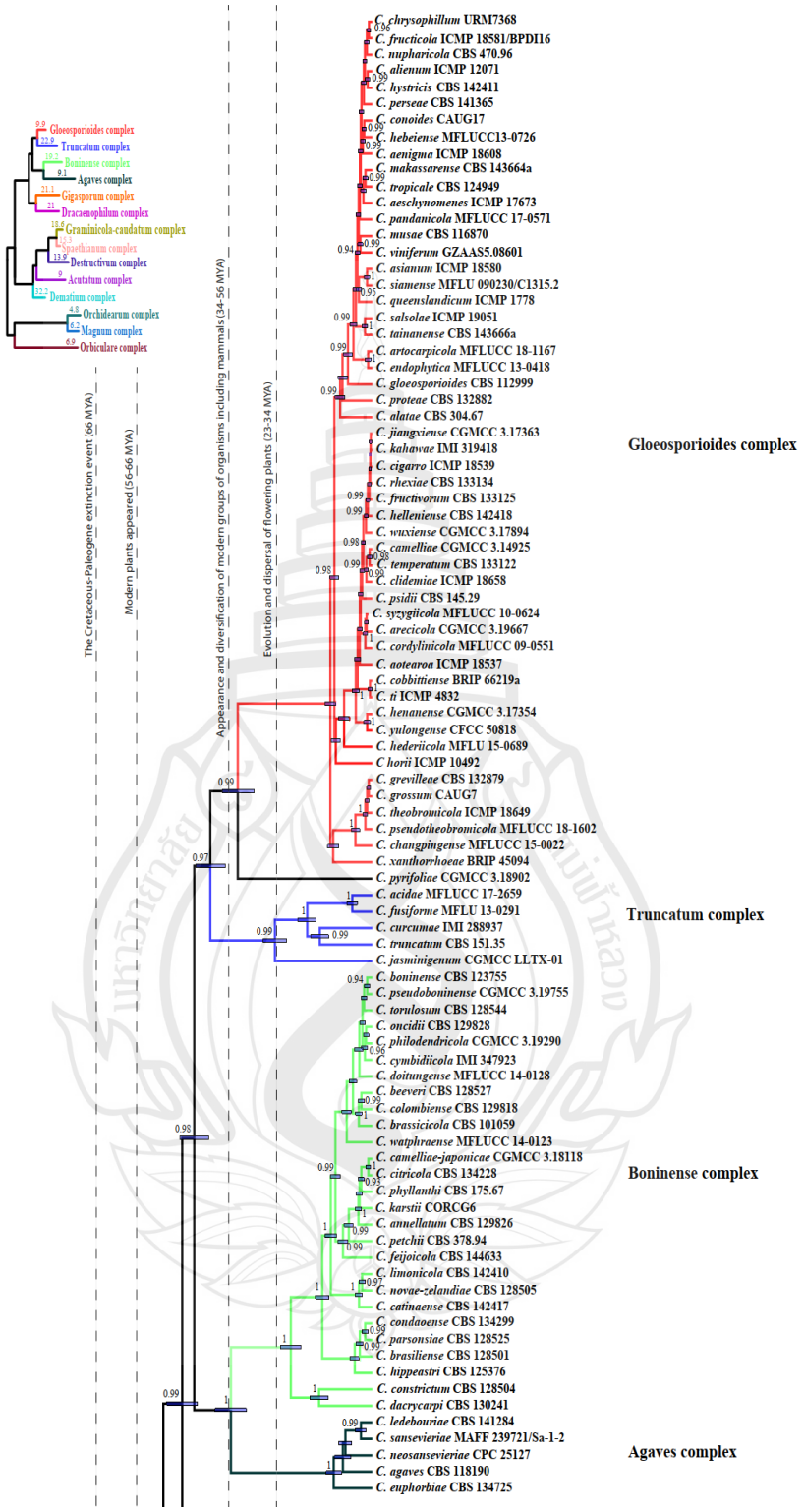


Figure 4.2 Maximum clade credibility tree of *Colletotrichum*

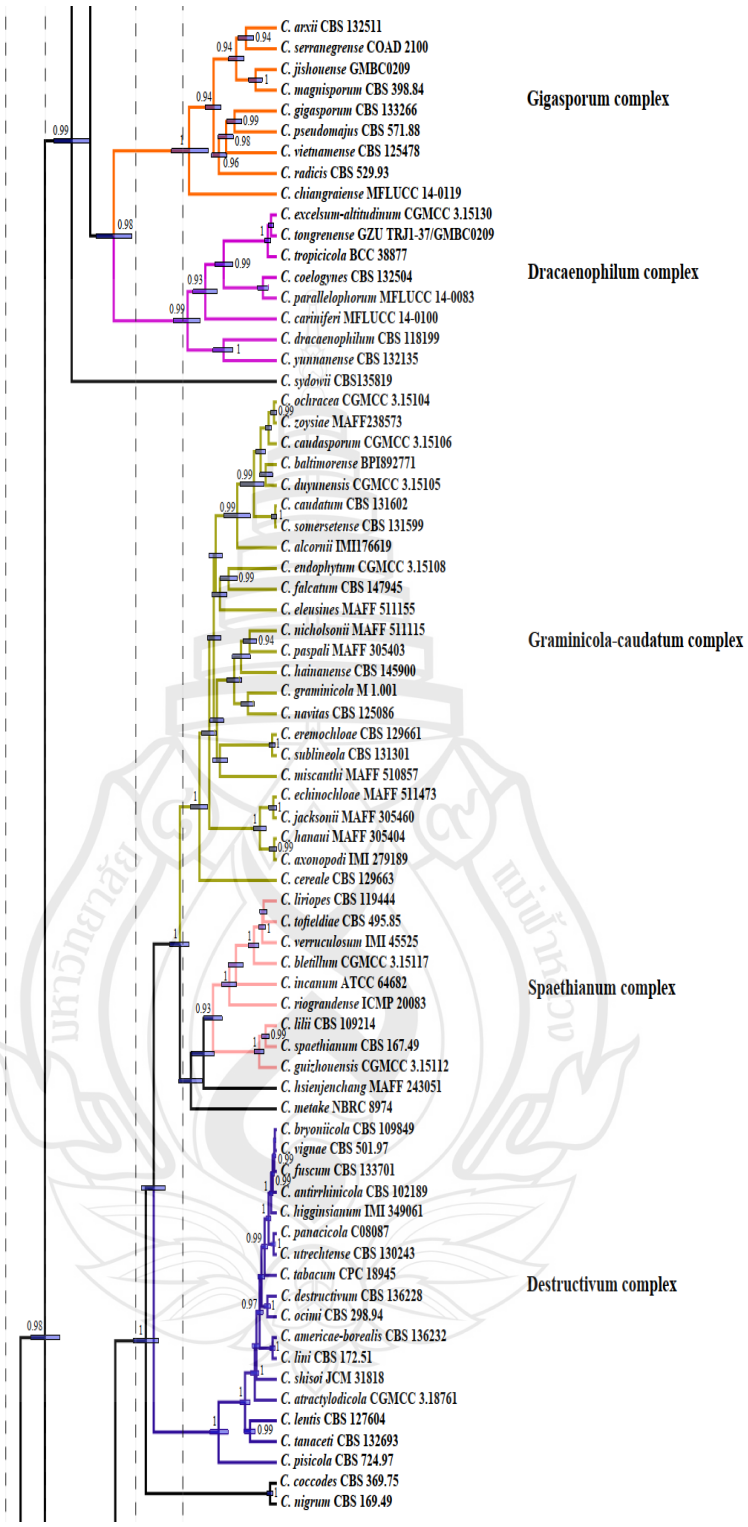
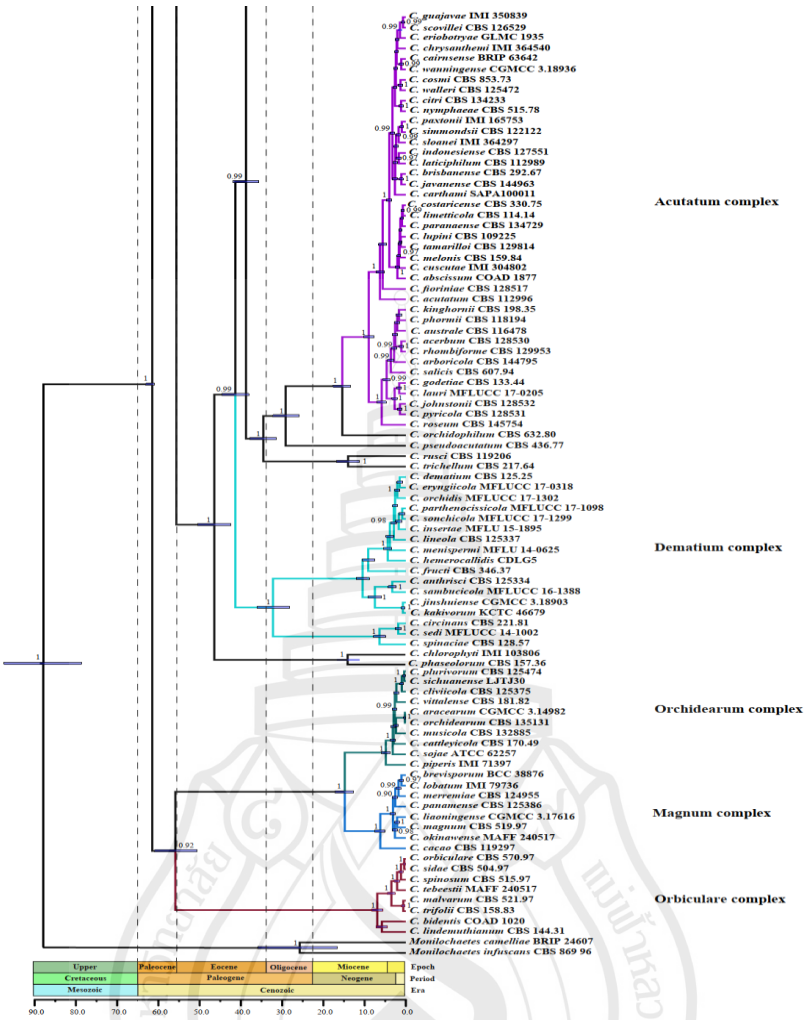


Figure 4.2 (continued)



Note Maximum clade credibility tree with divergence time estimates using BEAST. The divergence time are shown in million years and the numbers at the nodes indicate posterior probabilities (BYPP) for node support. Bars correspond to the 95% highest posterior density (HPD) intervals. The figure in the upper left corner represents the placement of all the complexes based on the maximum clade credibility tree and the estimated crown ages for all the complexes are shown in MYA. Each complex is represented by a different colour. Geological time scales are given at the base, together with scale in MYA [147].

Figure 4.2 (continued)

4.3.4 Larger Dataset Results

The larger concatenated dataset consisted of 555 taxa and sequences which did not cluster with their respective types based on all three phylogenetic analyses were removed. The final dataset consisted of 517 taxa with 821/2289 conserved sites, 1412/2289 variable sites and 1236/2289 were parsimony informative. The parsimony analysis of the dataset yielded one most parsimonious tree (Figure S4) out of 1000 (CI = 0.273, RI = 0.920, RC = 0.251, HI = 0.727, Tree Length = 10334). The best scoring RAxML tree had a final likelihood value of -55865.238686. With few exceptions, all three phylogenetic analyses based on the larger dataset resulted in clades with similar topology. The truncatum complex formed a sister clade to the gloeosporioides complex in the maximum likelihood analysis and Bayesian Inference analysis (99% MLBT/1.00 BYPP), but it formed a sister clade to the boninense and new species complex in the maximum parsimony analysis (87% MPBT).

Phylogenetic analyses based on the larger dataset was similar to the multi-locus datasets, except for the gigasporum and dracaenophilum complexes, as well as for few taxa from the graminicola complex. In the maximum parsimony analysis, *C. hainanense* formed a basal clade to the caudatum complex (100% MPBT). In the maximum likelihood analysis and Bayesian Inference analysis, *C. eremochloae* and *C. sublineola* formed a basal clade to the caudatum complex (100% MLBT/1.00 BYPP). The taxonomic placement of the gigasporum and dracaenophilum complexes did not differ in the different phylogenetic analyses based on the larger dataset. *Colletotrichum hsienjenchang* clustered with *C. metake* in the maximum likelihood and the Bayesian inference analyses (51% MLBT/0.70 BYPP). In the maximum parsimony analysis, *C. metake* formed a sister taxon to the graminicola complex with low support and *C. hsienjenchang* formed a sister taxon to the spaethianum complex with low support. The caudatum complex formed an inner clade in the graminicola complex based on all phylogenetic analyses of the larger dataset (100% MLBT/87% MPBT/1.00 BYPP). *Colletotrichum agaves*, *C. euphorbiae*, *C. ledebouriae*, *C. neosansevieriae* and *C. sansevieriae* formed a new complex in the maximum parsimony, maximum likelihood and the Bayesian inference analyses with strong support (100% MLBT/100% MPBT/1.00 BYPP). This complex formed a sister clade to the boninense complex in all phylogenetic analyses. *Colletotrichum parallelophorum* formed a sister taxon to *C.*

coelogynes species in the dracaenophilum complex (100% MLBT/100% MPBT/1.00 BYPP). *Colletotrichum cariniferi* clustered in the dracaenophilum complex with strong support in the Bayesian inference analysis, forming a basal clade to *C. parallelophorum* and *C. coelogynes* (58% MLBT/0.98 BYPP) whereas *C. axonopodi* formed a sister taxon to *C. hanaui* in the graminicola complex in all phylogenetic analyses (81% MLBT/0.86 BYPP).

The mPTP method delineated a higher number of entities (58 entities) in the larger dataset (Figure 4.3) compared to the multi-locus dataset. Compared to the multi-locus dataset, mPTP did not recover *C. hsienjenchang, C. metake* and the spaethianum complex as part of the caudatum and graminicola complexes in the larger dataset. In the larger dataset, *C. coelogynes* was not recovered as part of the dracaenophilum complex as compared to the multi-locus dataset. All the species in the gigasporum, magnum, orbiculare and orchidearum complexes were identified as distinct entities. The remaining complexes were divided into several entities (acutatum complex into 3 entities; boninense 8; dematium 2; destructivum 5; dracaenophilum 4; gloeosporioides 8; spaethianum 6; truncatum into 5). All the species from the caudatum and graminicola complexes including *C. axonopodi* were recovered as conspecific. *Colletotrichum sansevieriae*, *C. ledebouriae*, *C. neosansevieriae*, *C. euphorbiae* and *C. agaves* were identified as one taxonomic unit in the larger dataset. *Colletotrichum parallelophorum* along with the dracaenophilum complex were recovered as conspecific.

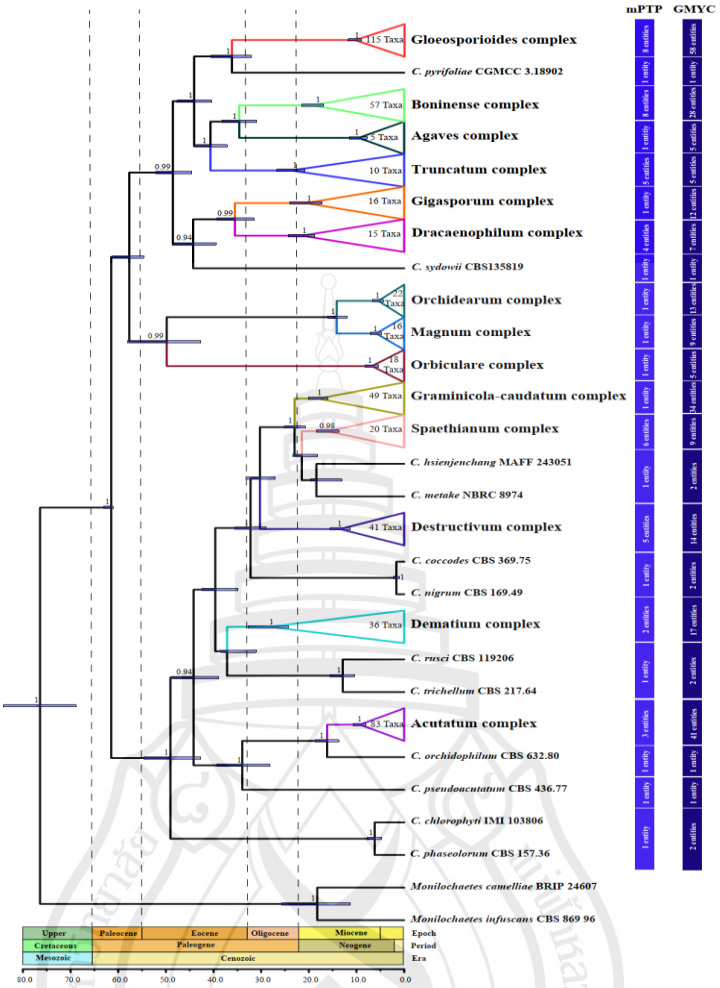
The GMYC method detected 279 entities in the larger dataset with the single and multiple threshold method (Figure 4 and S5), which is over twice the number of entities detected in the multi-locus dataset. In the larger dataset, *C. axonopodi* was not recovered as part of the graminicola complex and *C. parallelophorum* was not recovered as part of the dracaenophilum complex. In the larger dataset, the GMYC model provided a better fit to the ultra-metric tree based on the likelihoods for the null (L_0) and GMYC (L_{GMYC}) models (single threshold: L_0: 1676.793, L_{GMYC}: 1688.774, likelihood ratio (LR): 23.96139; multiple threshold: L_0: 1676.793, L_{GMYC}: 1707.259, LR: 60.93233). All the complexes were divided into several entities (acutatum into 41 entities; agaves 5; boninense 28; caudatum 10; dematium 17; destructivum 14; dracaenophilum 7; gigasporum 12; gloeosporioides 58; graminicola 24; magnum 9;

orbiculare 5; orchidearum 13; spaethianum 9; truncatum into 5 entities). All the singleton species were recovered as conspecific.

The maximum clade credibility tree (MCC) tree based on the larger dataset resulted in similar estimated crown ages as the MCC tree for the multi-locus dataset of type species with the exception of the truncatum complex and few taxa from the graminicola complex. In the larger dataset, the truncatum complex formed a sister clade to the agaves/boninense complexes (1.00 BYPP) and the taxa from the graminicola complex (*C. endophytum*, *C. eremochloae*, *C. falcatum* and *C. sublineola*) formed a basal clade to the caudatum complex. The topology of the maximum clade credibility (MCC) tree from the BEAST analysis of the larger dataset (Figure 4.3) was generally congruent with the result from the maximum likelihood, maximum parsimony and Bayesian inference analysis with some exceptions. The gigasporum and dracaenophilum complexes formed a sister clade to the gloeosporioides and boninense/sansevieriae/truncatum complexes in the MCC tree (0.99 BYPP). The caudatum complex formed an inner clade in the graminicola complex in the MCC tree. Three singleton taxa clustered in species complexes based on the MCC tree. *Colletotrichum parallelophorum* formed a sister taxon to *C. coelogynes* species in the dracaenophilum complex with strong posterior probability (1.00 BYPP). *Colletotrichum cariniferi* clustered in the dracaenophilum complex with strong support, forming a basal clade to *C. parallelophorum* and *C. coelogynes* (1.00 BYPP) and *C. axonopodi* formed a sister taxon to *C. hanaui* in the graminicola complex (0.90 BYPP).

The estimated crown age based on the larger dataset was similar to the multi-locus dataset (Table 4.1). The crown age of the caudatum complex was estimated at 5.8 MYA, spaethianum complex at 16 MYA, destructivum complex at 13.4 MYA, acutatum complex at 9.3 MYA, dematium complex at 28 MYA, gloeosporioides complex at 10.25 MYA, truncatum complex at 23.7 MYA, boninense complex at 19.1 MYA, gigasporum complex at 20.6 MYA, dracaenophilum complex at 21.4 MYA, orchidearum complex at 5.3 MYA, magnum complex at 5.8 MYA and the orbiculare complex at 6.7 MYA. *Colletotrichum sansevieriae*, *C. ledebouriae*, *C. neosansevieriae*, *C. euphorbiae* and *C. agaves* formed a separate complex with a crown age of 9.5 MYA. The stem age of this complex and the boninense complex was estimated at 34.5 MYA. The caudatum and graminicola complex (including the singleton *C. axonopodi*)

diverged at 18 MYA. The stem age of the gigasporum and dracaenophilum complex (including the singleton *C. parallelophorum* and *C. cariniferi*) was estimated at 35.5 MYA. The orchidearum and magnum complex diverged at 14.1 MYA. The orbiculare complex diverged from the orchidearum and magnum complex at 48.9 MYA.



Note Maximum clade credibility tree of the larger dataset with divergence time estimates using BEAST. The node represents the calibration point and the divergence time are shown in million years. Numbers at the nodes indicate posterior probabilities (BYPP) for node support. Bars correspond to the 95% highest posterior density (HPD) intervals. Each complex is represented by a different colour and the number of taxa (including the type taxa) in each complex is indicated. The columns present the results of mPTP and GMYC based on the larger dataset. Geological time scales are given at the base, together with scale in MYA [147].

Figure 4.3 Maximum clade credibility tree of the larger dataset

Table 4.1 Estimated crown and stem ages for all the complexes based on multi-locus and larger dataset. The median and the 95% Highest Posterior Density are provided in MYA

	Multi-locus dataset		Larger dataset	
Complexes	**Crown age**	**Stem age**	**Crown age**	**Stem age**
Acutatum	9 (7.7-10.3)	15.4 (13.4-17.6)	9.3 (8-10.7)	16.1 (13.7-18.7)
Agaves	9.1 (7.3-10.9)	33.3 (29.6-37)	9.5 (7.7-11.5)	34.5 (31-38.2)
Boninense	19.2 (16.8-21.6)	33.3 (29.6-37)	19.1 (16.9-21.5)	34.5 (31-38.2)
Graminicola-caudatum	18.6 (16.5-20.8)	23.3 (21-25.8)	18 (16.1-20.1)	23 (20.7-25.1)
Dematium	32.2 (28.2-36)	41.3 (38-44.6)	28 (24.2-32.8)	34.6 (31.1-38.6)
Destructivum	13.9 (11.7-16)	29.5 (26.7-32.4)	13.4 (11.4-15.6)	30 (27.1-33.2)
Dracaenophilum	21 (18.3-24.8)	39.1 (34.7-43.3)	21.4 (18.8-24.3)	35.5 (31.5-39.3)
Gigasporum	21.1 (16.4-25.3)	39.1 (34.7-43.3)	20.6 (17.12-24)	35.5 (31.5-39.3)
Gloeosporioides	9.9 (8.6-11)	31.7 (27.9-35.3)	10.25 (9-11.7)	36.1 (32.1-40.5)
Magnum	6.2 (5-7.3)	14.8 (12.7-17)	5.8 (4.7-7)	14.1 (12-16.1)
Orbiculare	6.9 (5.6-8.3)	55.8 (50.5-60.9)	6.7 (5.4-8.1)	48.8 (42.7-58)
Orchidearum	4.8 (3.8-5.9)	14.8 (12.7-17)	5.3 (4.3-6.6)	14.1 (12-16.1)
Spaethianum	15.3 (13-17.6)	17.5 (14.9-20.5)	16 (13.7-18.4)	20.7 (18.1-23.4)
Truncatum	22.9 (20-25.7)	38.2 (34.5-41.6)	23.7 (20.8-26.7)	40.6 (37.1-44)

4.4 Discussion

In this study, we evaluated the phylogenetic significance of five markers for species delineation within each species complex. The highest percentage of parsimony informative sites was recovered from the *GAPDH* gene (69%), followed by *ACT* (60%), *TUB2* (50%), the multi-locus dataset (49%), *CHS-1* (36%) and the ITS region (33%). Phylogeny based on the ITS marker effectively resolved the placement of all the taxa up to complex level, except *C. cereale* and *C. orchidis* which clustered in the spaethianum and destructivum complexes respectively. However, the placement of these two taxa can be resolved by using the multi-locus dataset. In the multi-locus dataset, *C. cereale* clustered in the graminicola complex and *C. orchidis* clustered in the dematium complex. The ITS region has been suggested to represent the universal

barcode for fungi [80] but with several concerns [81] and a database of annotated ITS region of plant pathogens was published in UNITE [278].

Phylogenetic analyses of the ITS, multi-locus and the larger dataset resulted in 14 strongly supported major clades, including a new complex, the agaves complex (Figure 4.1). This is in accordance to previous studies for 13 of the complexes (excluding the agaves complex) [52, 74, 249]. The caudatum complex formed a strongly supported inner clade in the graminicola complex based on phylogenetic analyses of the ITS, multi-locus dataset and the larger dataset which is also supported by previous studies [249]. Based on phylogenetic analyses of the ITS, multi-locus and larger dataset, three singleton taxa *C. axonopodi*, *C. cariniferi* and *C. parallelophorum* clustered in species complexes with strong support.

Five of the 13 markers that have been used in previous studies for species delineation in *Colletotrichum* were selected in this study. These markers were selected based on the availability of data especially for the type species and these represent the minimal set of markers to accurately delineate species in any of the species complexes. The intergenic region of DNA lyase (*apn2*) and mating type (*MAT1-2-1*) genes (*ApMat*) and Glutamine synthetase (*GS*) genes, which are important markers for the gloeosporioides complex [102, 271] were not included due to the lack of data available for isolates from complexes other than the gloeosporioides complex. The *GAPDH* and *TUB2* were the most informative markers for seven species complexes which is in accordance with previous studies for the acutatum and destructivum complexes [256]. However, the performance of the most informative marker was not the same for all the complexes. The *GAPDH* gene for example was the most informative marker for the gigasporum, orbiculare and truncatum complexes. The *TUB2* marker was the most informative for the gloeosporioides, graminicola and orchidearum complexes. The *GAPDH*, *ACT* and *TUB2* markers were all equally informative for the caudatum and the new complex. However, the assessment of the phylogenetic significance of the different markers is hindered by the lack of data, for example, the *CHS-1* marker is not available for the caudatum complex and the *GAPDH* marker is missing for most of the species in the graminicola complex. Therefore, the phylogenetic significance of these markers could not be assessed.

The mPTP method has been used for species delineation in several organisms with limited phenotypic variation [102, 279]. In this study, mPTP detected 26 taxonomic units in the multi-locus dataset compared to 58 entities in the larger dataset. In the multi-locus dataset, all the species from the caudatum, graminicola, and spaethianum complexes as well as the singletons *Colletotrichum axonopodi*, *C. hsienjenchang, C. metake* were recovered as conspecific. In the larger dataset, all the species from the caudatum and graminicola complexes including *C. axonopodi* were recovered as conspecific. Five singleton taxa *C. agaves, C. euphorbiae, C. ledebouriae*, *C. neosansevieriae* and *C. sansevieriae* were identified as one taxonomic unit in the multi-locus and the larger dataset. *Colletotrichum parallelophorum* was identified as part of the dracaenophilum complex in both datasets. In the larger dataset, *C. axonopodi* and the graminicola complex were identified as one entity. The results from mPTP therefore provides further support for the new complex. It also supports the taxonomic placement of *C. axonopodi, C. parallelophorum* and the placement of the caudatum complex in the graminicola complex.

Coalescent based approaches such as GMYC have been crucial for identifying cryptic species in numerous organisms [102, 279]. The GMYC approach detected 97 entities in the multi-locus dataset compared to 279 entities in the larger dataset. In the larger dataset, GMYC showed a tendency of over-splitting in some cases compared to phylogenetic and mPTP analyses. Over-splitting has been observed in some cases based on previous studies [28]. Based on low congruence between the GMYC result of the larger dataset and the phylogenetic analyses, we refer to the GMYC results from the multi-locus only. *Colletotrichum axonopodi* and the graminicola complex were recovered as one taxonomic unit whereas *C. parallelophorum* and the dracaenophilum complex were recovered as conspecific and two taxa from the new complex were recovered as conspecific. The results from GMYC based on the smaller dataset were in accordance with phylogenetic and mPTP analyses, thereby supporting the placement of *C. axonopodi, C. parallelophorum* and two taxa from the new complex.

Our study provides a global evolutionary picture of all the species in *Colletotrichum* and provides an estimated divergence time of all the complexes. The topology of the MCC tree based on both datasets yielded similar results except for three species from the graminicola complex. The caudatum complex formed a strongly

supported inner clade in the graminicola complex based on both datasets which is in accordance with phylogenetic analyses, mPTP and GMYC results. The divergence time estimates for the crown age of the complexes were similar in both datasets except for the caudatum complex. The MCC tree supported the phylogenetic placement of three singleton taxa (*C. axonopodi*, *C. cariniferi* and *C. parallelophorum* within species complexes. The MCC tree based on both datasets strongly supported a new species complex with an estimated divergence time of 9.5 MYA. The MCC tree based on both datasets resulted in 14 strongly supported major clades including the new species complex. The estimated divergence time for the complexes varied from approximately 4.8 MYA in the orchidearum complex to 32.2 MYA in the dematium complex. The estimated divergence time of the new complex fits within the range of the divergence time of the complexes in *Colletotrichum*, which provides further support for the agaves complex.

The evolutionary estimates from this study were congruent with divergence estimates from previous studies. Hacquard et al. [272] estimated the divergence time based on a penalized likelihood method of genome sequence data using the crown age of *Pezizomycotina*, *Sordariomycetes* and *Cordyceps-Metarhizium* divergence as the calibration points. They estimated that *C. incanum* and *C. tofieldiae* diverged approximately 8.8 MYA compared to 8–11 MYA in our study. Liang et al. [232] estimated the divergence time of several complexes based on a penalized likelihood method of genome sequence data using the crown age of *Colletotrichum*, *Sordariomycetes*, and *Sordariomycetes-Leotiomycetes* crown as the calibration points and the estimates were based on four calibration schemes. In the gloeosporioides complex, *C. fructicola* and *C. gloeosporioides* diverged approximately 3.8–5.1 MYA compared to 4.6–8.6 MYA in our study. In the graminicola complex, *C. graminicola* and *C. sublineola* diverged approximately 11–15 MYA compared to 12–16 MYA in our study. In the spaethianum complex, *C. incanum* and *C. tofieldiae* diverged approximately 7.5–11 MYA compared to 8–11 MYA in our study. In the acutatum complex, *C. salicis, C. fioriniae, C. nymphaeae* and *C. simmondsii* diverged approximately 8–10.9 MYA compared to 7.7–10.3 MYA in our study. The most recent common ancestor (MRCA) of the acutatum, graminicola, spaethianum and destructivum complex was estimated at 37–49 MYA compared to 35–41 MYA in our

study. The MRCA of the graminicola and spaethianum complex was estimated at 19–26 MYA compared to 21–25 MYA in our study. The stem age of orbiculare complex was estimated at 47–60 MYA compared to 42–60 MYA in our study. Lelwala et al. [280] estimated the divergence time based on a penalized likelihood method of genome sequence data using the crown age of *Leotiomycetes-Sordariomycetes*, crown age of *Sordariomycetes* and *Colletotrichum* crown as the calibration points. In the destructivum complex, *C. higginsianum* and *C. tanaceti* diverged around 9.9 MYA compared to 7.6 MYA in our study. In the gloeosporioides complex, *C. fructicola* and *C. gloeosporioides* diverged around 6.1 MYA compared to 7 MYA in our study. *Colletotrichum graminicola* and *C. sublineola* diverged around 15.8 MYA compared to 14.3 MYA in our study. In the acutatum complex, *C. fioriniae*, *C. nymphaeae*, *C. salicis* and *C. simmondsii* diverged around 10.9 MYA compared to 9 MYA in our study. The MRCA of the acutatum, graminicola, spaethianum and destructivum complex was estimated at 48.9 MYA compared to 38.7 MYA in our study. The MRCA of the graminicola, destructivum and spaethianum complex was estimated at 34.7 MYA compared to 29.5 MYA in our study.

Our results were therefore congruent with divergence estimates from previous studies which have used several calibration points, genome sequence data and a penalized likelihood method compared to the Bayesian analysis of five loci in our study [232, 272, 280]. Achieving congruent results from different methodologies therefore provide reliable support for divergence time estimation in our study. The evolutionary tree was calibrated by setting the crown age of *Colletotrichum* at minimum 61 MYA based on the fossil *Protocolletotrichum deccanensis.* The estimated crown age of *Colletotrichum* in our study was 61-63 MYA which in agreement to previous studies which were based on several calibration points [232]. Divergence time estimates can provide an insight in the evolutionary history of taxa, but at intraspecific divergence, it is still difficult to propose any taxonomic rearrangement due to overlap in estimated age.

Based on the resulting chronogram, the estimated crown age of *Colletotrichum* lies within the Paleocene epoch of the Paleogene period (61-63 MYA) which coincides with the period when modern angiosperm appeared [281]. The Cretaceous-Paleogene extinction event resulted in the disappearance of over 50% of plant species, thus causing

a loss of plant species diversity [282–283]. The conditions of high humidity and reduced solar insolation after the extinction event favoured an increase of saprobic fungi which flourished on the detritus [284]. *Colletotrichum* species can occur as endophytes, pathogens and saprobes and can switch lifestyle from endophytes to pathogens as a result of host senescence or wounding [284]. For example, *C. fructicola* has been isolated as an endophyte and pathogen [285] with an estimated divergence time of 0.78 MYA. *Colletotrichum endophytica* has been isolated as an endophyte and saprobe [284] with an estimated divergence time of 0.98 MYA. *Colletotrichum dematium* has been recorded as an endophyte, pathogen and saprobe [256] with an estimated divergence time of 1.49 MYA. This suggests that the endophytic phase is common to all species and other lifestyles could have evolved from this. The fossil *Protocolletotrichum* occurred as leaf spot disease during the Paleocene epoch of the Paleogene period which demonstrates that the pathogenic strategies of *Colletotrichum* were already well established [271].

The different complexes started to evolve at 28.2-36 MYA which lies within the Oligocene-Eocene epoch of the Paleogene period. The warm climate at the beginning of the Paleogene period favoured dense forest plants, then the humid subtropical conditions favoured an increase in the prevalence of several plants and grasses. As a result of evolution and dispersal of several plants including flowering plants during the Oligocene period (23-34 MYA), it can be hypothesised that *Colletotrichum* species diversified to adapt to various hosts. The Eocene epoch witnessed the appearance and diversification of several modern groups of organisms including mammals and the late Oligocene was linked to the expansion of grazing animals which could have facilitated the subsequent diversification of fungal species [286]. The earliest primitive stone tools were dated at 3.3 MYA which could be attributed to hominin lineage and the earliest stone tools associated with *Homo* were estimated at 2.58 MYA [287]. Stone tools were used for animal butchery as well as for plant processing at 2 MYA. At around 2 MYA, key adaptive behaviours were recorded in *Homo* including changes in body size and the ability of long-distance running [288]. The new mobility allowed humans to explore new lands and allowed long range transport of materials and food as resources became scarce in their immediate environment. It can therefore be hypothesised that during this period the lack of food supply also led to the establishment of agricultural practices and

monocropping although the earliest indication of cultivation is estimated at 23,000 years ago [289]. Intensive disturbance to the environment caused by the onset of cultivation could be associated with the further diversification of *Colletotrichum* species.

The morphology of the singleton *C. axonopodi*, *C. parallelophorum* and *C. cariniferi* were compared to the graminicola and dracaenophilum complexes respectively. *Colletotrichum axonopodi* (IMI279189) was isolated from *Axonopus affinis* and it is morphologically similar to grass-associated taxa in *Colletotrichum* [259]. It is a pathogen which is characterised by falcate conidia. The presence of widely falcate conidia is a characteristic feature of the graminicola complex and most of its species are important pathogens [256]. *Colletotrichum cariniferi* (MFLUCC 14–0100) was isolated as an endophyte from the stems of *Dendrobium cariniferum*. It has ellipsoidal to cylindrical conidia, with rounded ends when mature. *Colletotrichum parallelophorum* (MFLUCC 14–0083) was also isolated as an endophyte from *Dendrobium* sp. This species has smooth-walled, cylindrical conidia with rounded ends [290]. The presence of cylindrical conidia with rounded ends is a characteristic feature of dracaenophilum complex [256]. Therefore, the morphological characters of *C. axonopodi*, *C. parallelophorum* and *C. cariniferi* correspond to the graminicola and dracaenophilum complexes respectively.

The morphology of all the taxa in the new complex (*C. agaves*, *C. euphorbiae*, *C. ledebouriae*, *C. neosansevieriae* and *C. sansevieriae*) was also compared. *Colletotrichum sansevieriae* (MAFF239721) causes leaf blight of *Sansevieria trifasciata* [291]. It forms straight, cylindrical, conidia with obtuse apex. *Colletotrichum agaves* is associated with foliar diseases of agave plants [116]. This species has hyaline, cylindrical, straight or slightly curved conidia with broadly rounded apex. *Colletotrichum euphorbiae* was isolated from the leaves of *Euphorbia* sp. [78]. It forms pale brown conidiogenous cells and hyaline to pale orange, cylindrical to clavate conidia with round end on one side and truncate end on the other. *Colletotrichum neosansevieriae* (CBS 139918) is associated with leaf spot disease of *Sansevieria trifasciata* [78]. This species has hyaline conidiogenous cells and subcylindrical, straight to slightly curved, hyaline conidia with obtuse apex. *Colletotrichum ledebouriae* (CBS 141284) is associated with anthracnose disease on *Ledebouria* [291].

It forms hyaline to pale brown conidiogenous cells and hyaline, straight, subcylindrical conidia with obtuse apex. Therefore, the species in this complex are mostly pathogens with a wide range of hosts and they are characterised by the presence of straight or slightly curved conidia with obtuse apices. This species complex is named following the name of the species which was described first and therefore, this monophyletic group of five closely related species is named as the *Colletotrichum* agaves complex.

In this study, we compare the species boundaries in *Colletotrichum* using different genomic approaches including phylogenetic analyses, evolutionary analyses and coalescent-based methods. The coalescent and evolutionary methods provided congruent results to the phylogenetic analyses for most of the complexes. All analyses based on the ITS region, multi-locus dataset and larger dataset recovered the caudatum and graminicola complexes as one. Therefore, we recommend that these two complexes should be treated as one. Based on our findings, *C. axonopodi* is accepted in the graminicola complex whereas *C. parallelophorum* and *C. cariniferi* are accepted in the dracaenophilum complex. Based on congruent results from different molecular approaches, we accept 248 species and the vast majority are classified in 14 species complexes (40 species in acutatum complex, 25 in boninense, 17 in dematium, 17 in destructivum, 8 in dracaenophilum, 9 in gigasporum, 52 in gloeosporioides, 24 in graminicola-caudatum, 8 in magnum, 8 in orbiculare, 9 in orchidearum, 5 in sansevieriae, 9 in spaethianum, 5 species in truncatum complex and 12 singleton taxa). We recommend multi-locus dataset for accurate species delineation in *Colletotrichum* as there is currently no single marker that can discriminate between species in all the complexes. Our study has identified the most informative marker for different complexes which must be included for species identification for each of the complexes. The implementation of coalescent based approaches supported our findings based on phylogenetic analyses. We therefore recommend the application of coalescent-based methodologies to delineate species in complex genus such as *Colletotrichum* as achieving congruent results across different genomic methods provides a robust framework to reliably elucidate species and species complex boundaries.

CHAPTER 5

MULTIGENE PHYLOGENETIC CHARACTERISATION OF *COLLETOTRICHUM ARTOCARPICOLA SP. NOV.* FROM *ARTOCARPUS HETEROPHYLLUS* IN NORTHERN THAILAND[4]

Abstract

A new species, *Colletotrichum artocarpicola,* on *Artocarpus heterophyllus* from Chiang Rai, Thailand, is introduced using both morphological and molecular approaches. Combined phylogenetic analysis of ITS, GAPDH, CHS-1, ACT and TUB2 sequence data demonstrate that *Colletotrichum artocarpicola* is a distinct species within the gloeosporioides species complex. The new species is illustrated and compared with related taxa, and evidence of its pathogenicity is provided.[1]

Keywords: 1 New Species, Molecular Phylogeny, Morphology, Saprobe, Pathogenicity

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I declare that my contribution to this chapter was 95%

5.1 Introduction

The genus *Colletotrichum* was introduced by Corda [247] and belongs to the family *Glomerellaceae* (*Glomerellales, Sordariomycetes*) [8, 20]. Species of this genus can occur as endophytes and saprobes [8, 42, 186, 248, 256], as well as plant and human pathogens [115, 138, 252]. It is worth noting that the same species can have different nutritional modes based on environmental conditions [249]. For example, *C. fructicola* is a pathogen on various hosts [257] but can also be found as a saprobe and an endophyte.

Species delimitation based on morphology alone is difficult due to overlapping morphological characters in asexual morphs and their sexual morphs are not often produced [60, 74]. Misunderstanding of their host-specific nature has also led to considerable confusion concerning the species concept [74, 249]. The application of a polyphasic approach including multi-loci sequence analyses, analyses of geographical, ecological and morphological data as suggested by Cai et al. [76] is needed to provide a better taxonomic resolution for this genus. Index Fungorum [30] lists 894 epithets (www.indexfungorum.org; accessed 5th August 2019) under the genus *Colletotrichum*, although there are fewer than 250 accepted species in 14 species complexes [249, 256]. We have been studying *Colletotrichum* in northern Thailand since 2007 and have identified 16 new species [8, 273]. Hyde et al. [168] predicted that there may be more *Colletotrichum* species in northern Thailand and can be discovered through extensive sampling. *Artocarpus heterophyllus,* commonly known as jack fruit, is important commercially and is a delicacy in Thailand. Several species of *Colletotrichum* have been recorded from *Artocarpus* species in various parts of the world (Table 5.1).

In this paper, we describe and illustrate a new *Colletotrichum* species, *C. artocarpicola*, identified by means of morphology and multi-loci phylogeny.

Table 5.1 Species of *Colletotrichum* associated with *Artocarpus* species

***Artocarpus* species**	***Colletotrichum* species**	**Country**	**References**
Artocarpus altilis	*Colletotrichum* sp.	Fiji, Kiribati, Samoa, Tonga	Dingley et al. (1981); Mckenzie (1996)
Artocarpus communis	*C. artocarpi*	Hawaii	Raabe et al. (1981)
Artocarpus communis	*C. gloeosporioides*	Dominican Republic, Myanmar	Ciferri (1961); Thaung (2008)
Artocarpus heterophyllus	*C. gloeosporioides*	China, Thailand	Zhuang (2001); Giatgong (1980)
Artocarpus heterophyllus	*C. fructicola* (*C. gloeosporioides* var. *minor*)	Australia	Simmonds (1966)
Artocarpus heterophyllus	*C. siamense*	Australia	James et al. (2014); Shivas et al. (2016)
Artocarpus incisa	*C. artocarpi*	Hawaii	Stevens (1925)
Artocarpus incisa	*C. gloeosporioides*	India	Mathur (1979)
Artocarpus incisus	*C. artocarpi*	Hawaii	Miller et al. (1960)
Artocarpus integrifolia	*C. gloeosporioides*	Brazil, India	Mendes (1998); Sarbhoy (1990)
Artocarpus sericicarpus	*C. siamense*	Australia	Shivas et al. (2016)
Artocarpus heterophyllus	*C. artocarpicola*	Thailand	This study

5.2 Materials and Methods

5.2.1 Sample Collection, Isolation and Identification

Strain RJF2 (MFLUCC 18–1167) was isolated from a dead root sample of *Artocarpus heterophyllus* collected by RS. Jayawardena from Chiang Rai, Thailand in 2018. Pure fungal colonies were obtained using the method given in Chomnunti et al. [292]. Germinating spores were transferred aseptically to Potato Dextrose Agar (PDA) and the cultures were incubated at 25 ℃ for 10 to 15 days with frequent observations. Fungal morphological characters were observed following the method

given in Cai et al. [76] using a stereo microscope (Zeiss Discovery v8) fitted with Axio Cam ERc5S and Leica DM2500 compound microscope attached with a Leica MC190 HD camera. All microscopic measurements were carried out using Tarosoft (R) Image Frame Work program and the images were processed with Adobe Photoshop CS6 version 13.0 software (Adobe Systems, USA). The type specimen was deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand and living cultures were deposited in the Culture Collection at Mae Fah Luang University (MFLUCC) and Kunming University Culture Collection (KUMCC). The new taxon was linked with Facesoffungi numbers (FoF) and Index Fungorum numbers [184].

5.2.2 DNA Extraction, PCR Amplification and DNA Sequencing

Total genomic DNA was extracted from fresh mycelium cultured from the margin of colonies on PDA plates. The internal transcribed spacer regions (ITS) of the rRNA gene and the genes coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1 (CHS-1), actin (ACT) and β-tubulin (TUB2) were amplified using primers given in Table 5.2.

PCR was performed using PCR mixtures containing TaKaRa Ex-Taq DNA polymerase 0.3µl, 12.5µl of 2 × PCR buffer with 2.5µl of dNTPs, 1µl of each primer, 9.2µl of double-distilled water and 100–500 ng of DNA template following the thermal cycling program described by Weir et al. [257]. All the PCR products were visualised by staining with ethidium bromide (EtBr) on 1.2% agarose electrophoresis gels [293] and successful PCR products were purified according to the manufacturer's instructions of a Qiagen purification kit (Qiagen, USA). DNA sequencing of the genes was conducted using same PCR primers by Sunbiotech Company, Beijing, China.

Table 5.2 Primers used in the study

Gene	Primer	Sequence (5'-3')	References
ITS	ITS 1	TCC GTA GGT GAA CCT GCG G	White et al. [239]
	ITS 4	TCC TCC GCT TAT TGA TAT GC	
GAPDH	GDF	GCC GTC AAC GAC CCC TTC ATT GA	Templeton et al. [294]
	GDR	GGG TGG AGT CGT ACT TGA GCA TGT	
CHS-1	CHS-79F	TGG GGC AAG GAT GCT TGG AAG AAG	Carbone & Kohn [295]
	CHS-345R	TGG AAG AAC CAT CTG TGA GAG TTG	
ACT	ACT-512F	ATG TGC AAG GCC GGT TTC GC	Carbone & Kohn [295]
	ACT-783R	TAC GAG TCC TTC TGG CCC AT	
TUB2	BT-2F	AAC ATG CGT GAG ATT GTA AGT	O'Donnell & Cigelnik [296]
	BT-4R	TAG TGA CCC TTG GCC CAG TTG	

5.2.3 Phylogenetic Analysis

The sequence data was assembled using BioEdit v. 7.2.5 [188] and subjected to a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest matches. Reference sequence data were downloaded and were automatically aligned using default settings in MAFFT v. 7 [http://mafft.cbrc.jp/alignment/server/, 297]. The combined dataset of the five gene regions was prepared and manually adjusted using BioEdit where necessary. PAUP v. 4.0b10 [191] was used to conduct the maximum parsimony analysis (MP). Gaps were treated as missing data and ambiguously aligned regions were excluded. Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. Maxtrees were set up to 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length [TL], Consistency Index [CI], Retention Index [RI], Rescaled Consistency Index [RC] and Homoplasy Index [HI]) were calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications resulting from maximum parsimony analysis. Kishino-Hasegawa tests (KHT) [192] were performed to determine whether the trees were significantly different. Bayesian Inference (BI) analysis was conducted using MrBayes v. 3.1.2 [193]. Six simultaneous Markov

chains were run for 1,000,000 generations and trees were sampled every 100th generation. The first 20% of generated trees representing the burn-in phase of the analyses were discarded and the remaining 80% of trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree. Phylograms were visualized with FigTree v1.4.0 program [194] and reorganized in Microsoft powerpoint and Adobe Illustrator CS5 (Version 15.0.0, Adobe, San Jose, CA). The alignment and tree are deposited in TreeBASE under accession number S25063. The fungal strains used for the phylogenetic analysis in this study are listed in Table 5.3.

Table 5.3 Taxa used for the phylogenetic analyses in this study. Ex-type/ex-epitype or reference strains are marked with an Asterix (*).

Taxon	Accession number	GenBank accession numbers				
		ITS	GAPDH	CHS-1	ACT	TUB2
Colletotrichum aenigma	ICMP 18608*	JX010244	JX010044	JX009774	JX009443	JX010389
C. aeschynomenes	ICMP 17673*	JX010176	JX009930	JX009799	JX009483	JX010392
C. alatae	ICMP 17919*	JX010190	JX009990	JX009837	JX009471	JX010383
C. alienum	ICMP 12071*	JX010251	JX010028	JX009882	JX009572	JX010411
C. aotearoa	ICMP 18537*	JX010205	JX010005	JX009853	JX009564	JX010420
C. artocarpicola	MFLUCC 18-1167*	MN415991	MN435568	MN435569	MN435570	MN435567
C. asianum	ICMP 18580*	FJ972612	JX010053	JX009867	JX009584	JX010406
C. boninense	CBS 123755*	JQ005153	JQ005240	JQ005327	JQ005501	JQ005588
C. camelliae	CGMCC 3.14925*	KJ955081	KJ954782	-	KJ954363	KJ955230
C. catinaense	CBS 142417*	KY856400	KY856224	KY856136	KY855971	KY856482
C. chengpingense	MFLUCC 15-0022*	KP683152	KP852469	KP852449	KP683093	KP852490
C. clidemiae	ICMP 18658*	JX010265	JX009989	JX009877	JX009537	JX010438
C. conoides	CAUG17*	KP890168	KP890162	KP890156	KP890144	KP890174
C. cordylinicola	ICMP 18579*	JX010226	JX009975	JX009864	HM470235	JX010440

Table 5.3 (continued)

Taxon	Accession number	GenBank accession numbers				
		ITS	GAPDH	CHS-1	ACT	TUB2
C. endophytica	MFLUCC 13-0418*	KC633854	KC832854	-	KF306258	-
C. endophytica	YN32-6	MH636511	MH681390	MH622453	MH622589	MH622721
C. endophytica	YN32-2	MH636509	MH681388	MH622451	MH622587	MH622719
C. endophytica	HN37-6	MH636453	MH681332	MH622395	-	MH622663
C. endophytica	HN37-2	MH636452	MH681331	MH622394	-	MH622662
C. endophytica	YN1A4	KU251561	KU252015	KU251909	KU251642	KU252169
C. endophytica	YN1A5	KU251560	KU252014	KU251908	KU251641	KU252168
C. endophytica	CAUG28	KP145441	KP145413	KP145385	KP145329	KP145469
C. fructicola	ICMP 18581*	JX010165	JX010033	JX009866	FJ907426	JX010405
C. fructivorum	Coll1414*	JX145145	-	-	-	JX145196
C. gloeosporioides	CBS 112999*	JQ005152	JQ005239	JQ005326	JQ005500	JQ005587
C. grevilleae	CBS 132879*	KC297078	KC297010	KC296987	KC296941	KC297102
C. hebeiense	MFLUCC 13-0726*	KF156863	KF377495	KF289008	KF377532	KF288975
C. henanense	CGMCC 3.17354*	KJ955109	KJ954810	-	KM023257	KJ955257
C. horii	ICMP 10492*	GQ329690	GQ329681	JX009752	JX009438	JX010450
C. hystricis	CPC 28153*	KY856450	KY856274	KY856190	KY856023	KY856532
C. jiangxiense	CGMCC 317363*	KJ955201	KJ954902	-	KJ954471	KJ955348
C. kahawae	ICMP 17816*	JX010231	JX010012	JX009813	JX009452	JX010444
C. ledongense	CGMCC 3.18888*	KX853165	KX893584	-	KX893576	KX893580
C. mangiferae-indicae	MFLUCC 18-1182*	MK629453	MK639363	MK639357	MK639359	MK639361
C. musae	ICMP 19119*	JX010146	JX010050	JX009896	JX009433	HQ596280
C. nupharicola	ICMP 18187*	JX010187	JX009972	JX009835	JX009437	JX010398

Table 5.3 (continued)

Taxon	Accession number	GenBank accession numbers				
		ITS	GAPDH	CHS-1	ACT	TUB2
C. pandanicola	MFLUCC 17-0571	MG646967	MG646934	MG646931	MG646938	MG646926
C. proteae	CBS 132882*	KC297079	KC297009	KC296986	KC296940	KC297101
C. pseudotheobromicola	JZB330119*	MG763975	MG812553	-	MG812544	MG812559
C. psidii	ICMP 19120*	JX010219	JX009967	JX009901	JX009515	JX010443
C. queenslandicum	ICMP 1778*	JX010276	JX009934	JX009899	JX009447	JX010414
C. rhexiae	Coll1026*	JX145128	-	-	-	JX145179
C. salsolae	ICMP 19051*	JX010242	JX009916	JX009863	JX009562	JX010403
C. siamense	ICMP 18578*	JX010171	JX009924	JX009865	FJ907423	JX010404
C. syzygicola	MFLUCC 10-0624*	KF242094	KF242156	-	KF157801	KF254880
C. temperatum	Coll883*	JX145159	-	-	-	JX145211
C. theobromicola	ICMP 18649*	JX010294	JX010006	JX009869	JX009444	JX010447
C. ti	ICMP 4832*	JX010269	JX009952	JX009898	JX009520	JX010442
C. tropicale	ICMP 18653*	JX010264	JX010007	JX009870	JX009489	JX010407
C. viniferum	GZAAS5.08601*	JN412804	JN412798	-	JN412795	JN412813
C. wuxiense	CGMCC 3.17894*	KU251591	KU252045	KU251939	KU251672	KU252200
C. xanthorrhoeae	ICMP 17903*	JX010261	JX009927	JX009823	-	JX010448

5.2.4 Genealogical Concordance Phylogenetic Species Recognition Analysis

The new species and its closely related species were analysed using the Genealogical concordance phylogenetic species recognition (GCPSR) model. A pairwise homoplasy index (PHI) [298] test was performed in SplitsTree4 [298] as described by Quaedvlieg et al. [228] to determine the recombination level within phylogenetically closely related species using a five-locus concatenated dataset. As significance is set at 0.05 level, if $P<0.05$, the null hypothesis is rejected and the alternate hypothesis is accepted that significant recombination was detected among the strains when applying PHI tests with the GCPSR model. The relationships between closely related species were visualised by constructing a split graph, using both the LogDet transformation and splits decomposition options (Figure 5.3).

5.2.5 Pathogenicity Assay

To test the pathogenicity of the new species, a wound and non-wound assay was conducted on the leaves of *Artocarpus heterophyllus*, *Carica papaya* and *Capsicum* sp. The samples were surface sterilized by washing in 75% ethanol for 1 min, then in 5% sodium hypochlorite for 1 min, followed by washing with distilled water three times, and air-dried on sterile filter paper. Six of the leaves of *Artocarpus heterophyllus*, *Carica papaya* and *Capsicum* sp were wounded by means of a sterilized blade (one wound per fruit), while six remained unwounded. A conidial suspension (1×10^6 conidial/mL) was applied on two wounded and two non-wounded leaves of *Artocarpus heterophyllus*, *Carica papaya* and *Capsicum* sp by using a dropper while the control samples were inoculated with sterilized water. Colonised mycelium plugs were applied on two wounded and two non-wounded samples and sealed with parafilm. Mycelium plugs were obtained from the periphery of actively growing fungal colonies cultivated on potato dextrose agar (PDA). Uncolonized PDA plugs were used for control inoculations. The inoculated samples and the control samples were incubated in a moist chamber at 28 °C with an 80% relative humidity until symptoms appeared. Lesion lengths were recorded and Koch's postulates were confirmed by re-isolating the inoculated fungus. The re-isolated fungus was identified based on cultural and morphological characters.

5.3 Results

5.3.1 Phylogenetic Analyses

The combined gene alignment for the gloeosporioides species complex comprised 52 taxa and 1812 characters including gaps with *C. boninense* (CBS 123755) and *C. catinaense* (CBS 142417) as the outgroup taxa. Parsimony analysis indicated the presence of 1221 constant characters, 215 variable characters and 376 parsimony-informative characters respectively. The parsimony analysis of the data matrix yielded a single most parsimonious tree (TL = 1096, CI = 0.684, RI = 0.787, RC = 0.539, HI = 0.316) which is presented in Figure 5.1. *Colletotrichum artocarpicola* is basal to *C. endophytica*, forming a separate lineage with high

bootstrap values and high Bayesian posterior probabilities (MP:92%, ML:83%, BYPP:1.00). Therefore, to accommodate this taxon, a new species is introduced. A PHI test revealed no significant recombination event between *C. artocarpicola* and its closely related taxa (Figure 5.3).

Table 5.4 Base pair difference between *C. artocarpicola* and different strains of *C. endophytica*

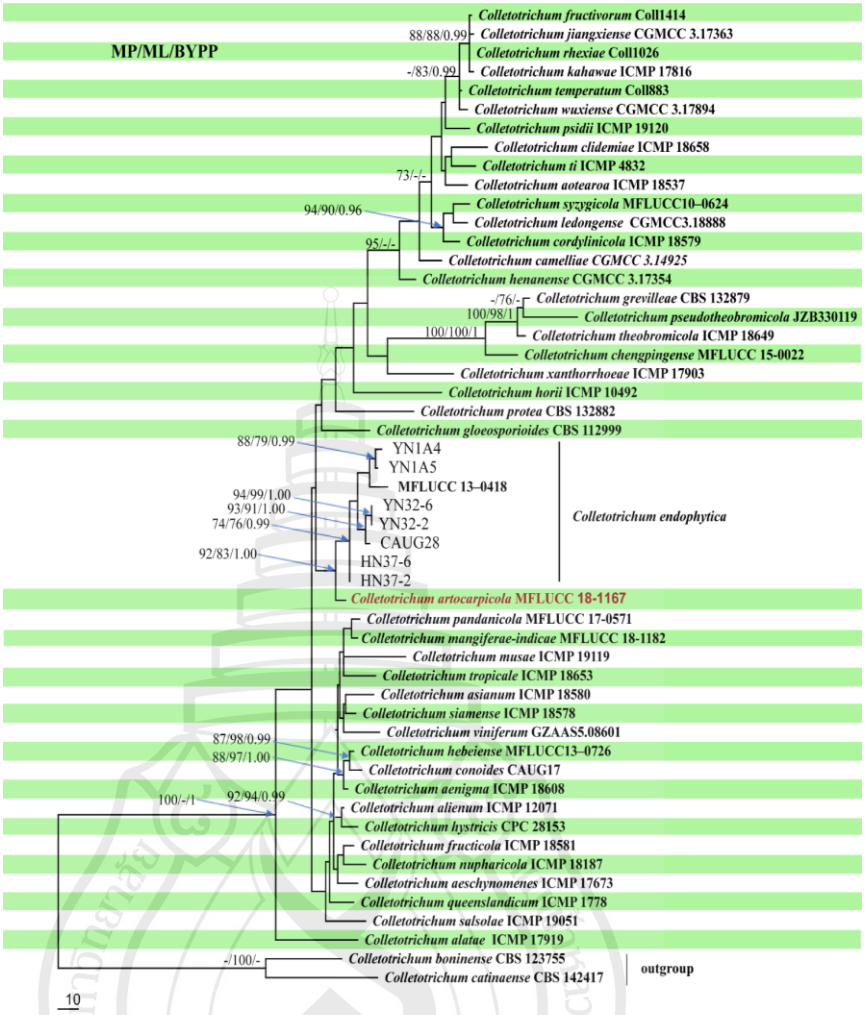
Taxa	Gene regions				
	ITS	**GAPDH**	**CHS-1**	**ACT**	**TUB2**
C. endophytica MFLUCC 13-0418	1/446	5/223	-	33/257	-
C. endophytica YN1A4	4/466	7/223	5/277	4/256	7/481
C. endophytica YN1A5	4/466	7/223	5/277	0/256	7/481
C. endophytica YN32-6	4/479	4/223	5/277	0/256	8/481
C. endophytica CAUG28	4/479	4/223	5/277	0/256	8/481
C. endophytica HN37-6	2/479	4/223	1/277	-	7/481
C. endophytica HN37-2	2/479	4/223	1/277	-	7/481

The base pair difference between *C. artocarpicola* and *C. endophytica* were compared as shown in Table 5.4. *Colletotrichum artocarpicola* differs from *C. endophytica* (MFLUCC 13-0418) by <1% bp difference in ITS, 2.24% bp difference in GAPDH and 12.84% bp difference in ACT. *Colletotrichum artocarpicola* was also compared with *C. endophytica* YN1A4 and YN1A5. The ITS gene regions showed <1% bp difference, GAPDH 3.14% bp difference, CHS-1 2.2% bp difference and TUB2 1.46% bp difference. The ACT gene region of *C. artocarpicola* was 100% similar to *C. endophytica* YN1A5, YN32-6, YN32-2, CAUG28 and it had 1.56% bp difference for *C. endophytica* YN1A4. *Colletotrichum endophytica* YN32-6, YN32-2 and CAUG28 differ from *C. artocarpicola* by <1% bp difference in ITS, 1.79% bp difference in GAPDH, 1.81% bp difference in CHS-1 and 1.66% bp difference in TUB2. The ITS gene region showed <1% bp difference, 1.79% bp difference in GAPDH, <1% bp difference in CHS-1 and 1.46% bp difference between *Colletotrichum artocarpicola* and *C. endophytica* HN37-6 and HN37-2.

The base pair difference between the type strain of *C. endophytica* and the different strains of *C. endophytica* were compared (Table 5.5) as these strains were segregated in two separate groups. *Colletotrichum endophytica* YN32-6 and YN32-2 differ from *C. endophytica* by 1.12% bp difference in ITS, 2.24% bp difference in GAPDH, 2.18% (YN32-6) and 1.75% (YN32-2) bp difference in ACT respectively. The ITS region of *C. endophytica* CAUG28 differ by 1.12% bp, 1.79% bp difference in GAPDH and 1.75% bp difference in ACT compared to *C. endophytica. Colletotrichum endophytica* HN37-6 and HN37-2 differ from *C. endophytica* by <1% bp in ITS and GAPDH gene region. The ACT gene regions are not available for *Colletotrichum endophytica* HN37-6 and HN37-2. These differences in the different gene regions for *Colletotrichum endophytica* YN32-6, YN32-2, HN37-6, HN37-2 and CAUG28 suggest why they did not cluster with the type of *C. endophytica*.

Table 5.5 Base pair difference between *C. endophytica* (MFLUCC 13-0418) and *C. endophytica* strains

Taxa	Gene regions				
	ITS	**GAPDH**	**CHS-1**	**ACT**	**TUB2**
Colletotrichum endophytica YN32-6	5/446	5/223	-	5/229	-
C. endophytica YN32-2	5/446	5/223	-	4/228	-
C. endophytica CAUG28	5/446	4/223	-	4/228	-
C. endophytica HN37-6	3/446	1/223	-	-	-
C. endophytica HN37-2	3/446	1/223	-	-	-



Note Phylogenetic tree generated by maximum parsimony analysis of combined ITS, GAPDH, CHS-1, ACT and TUB2 sequence data of *Colletotrichum gloeosporioides* species complex. The tree is rooted with *Colletotrichum boninense* (CBS 123755) and *C. catinaense* (CBS 142417). Maximum parsimony and RAxML bootstrap support value ≥70% (BT) as well as Bayesian posterior probabilities ≥0.90 (PP) are shown respectively near the nodes. The scale bar indicates 10 changes. Ex-type strains are in bold and the isolate of this study is in red.

Figure 5.1 Phylogenetic analysis of *Colletotrichum gloeosporioides* species complex

5.3.2 Description and Taxonomy

Colletotrichum artocarpicola Bhunjun, Jayawardena, Jeewon & K. D. Hyde, *sp. nov*.

Index Fungorum number: IF556758; Facesoffungi number: FoF 06375, Figure 5.2

Etymology: The name refers the host from which this fungus was isolated.

Holotype: MFLU 18–1167

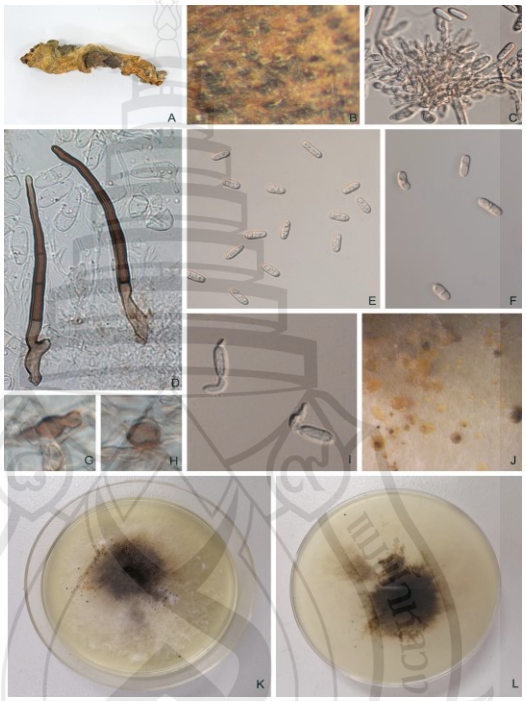
Saprobic on decaying root of *Artocarpus*. Asexual morph: *Conidiomata* 80–110 μm ($\overline{x}$ = 96 μm, n=10) diam., black, circular to oval, submerged, aggregated. *Setae* straight or bent, scarce, brown, sometimes becoming paler towards the apex, opaque, smooth-walled, base pale brown and irregular in shape, 1-2-septate, 61–67 *μm* long, 2–5 *μm* diam. ($\overline{x}$ = 64×3 μm, n=3), tip acute. *Conidiophores* hyaline, cylindrical or clavate, smooth-walled, simple, occurring in densely arranged clusters. *Conidiogenous cells* hyaline, straight to flexuous, cylindrical to slightly inflated, periclinal thickening not visible. *Conidia* 10–17×3–7 μm ($\overline{x}$ = 12×6 μm, n=40) unicellular, hyaline, often 1- septate, smooth, cylindrical, sometimes slightly ovoid with rounded ends. *Appressoria* 5–16×4–15 μm ($\overline{x}$ =9×9 μm, n=20), solitary to aggregated, medium to dark brown, mostly globose. Sexual morph: not observed.

Culture characters: Colonies on PDA attaining 25 mm diam. after seven days of incubation at 25 ℃ with flat irregular margin, aerial mycelium sparse with conidial masses scattered around the dark-grey region, off-white to grey, reverse initially white, dark grey at the center, turning grey to black with age.

Material examined: THAILAND, Chiang Rai, Thailand, on dead root sample of *Artocarpus heterophyllus*, 25 July 2018, RS. Jayawardena, RJF2, MFLU 18–1167 (holotype), MFLU 18–1167 (ex-type living culture).

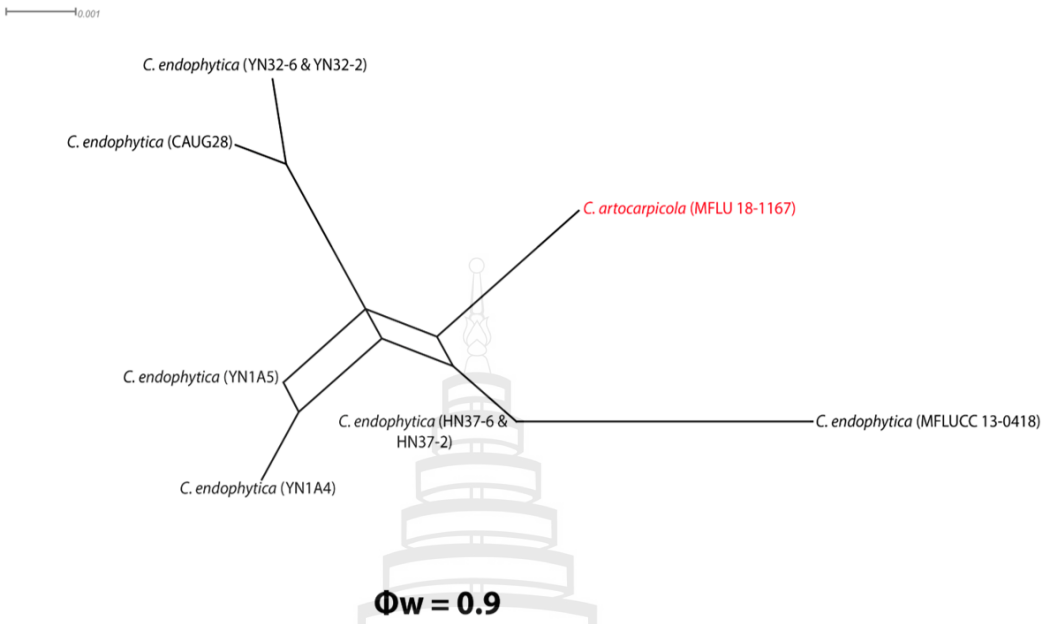
Notes. The new taxon *Colletotrichum artocarpicola* is morphologically similar to *C. endophytica* as they share cylindrical or clavate conidiophores arranged in clusters and unicellular, hyaline, cylindrical sometimes slightly ovoid conidia with rounded ends. However, the colonies of *C. endophytica* (MFLUCC 13-0418) on PDA are dense and raised while the colonies of *C. artocarpicola* are sparse and flat. The size of appressoria is slightly different between the two species (*C. endophytica* 8–

12×4–8 µm *vs C. artocarpicola* 5–16×4–15 µm) [172]. *Colletotrichum artocarpicola* differs from *C. endophytica* due to slight differences in the size of conidia (*C. endophytica* 13–19×5–6 µm *vs C. artocarpicola* 10–17×3–7 µm) [172]. Germinating conidia of *C. artocarpicola* have a septum while this was not observed in *C. endophytica.*



Note *Colletotrichum artocarpicola* (MFLUCC 18-1167 - holotype). A, B. Appearance of conidiomata on the root of jack fruit. C. Conidiophores. D. Setae. E. Conidia. F. Conidia with septum (closer to germination phase). G, H. Appressoria. I. Germinating conidia. J. Conidiomata on PDA. K. Upper view of culture (2 weeks old). L. Reverse view of culture. M. Induced symptom on banana after 7 days. Scale bars: B = 200 µm, C, E-F, I = 50 µm, D = 20 µm, G = 25 µm, H = 20 µm, J = 500 µm.

Figure 5.2 Morphological characters of *Colletotrichum artocarpicola* (MFLUCC 18-1167 - holotype)

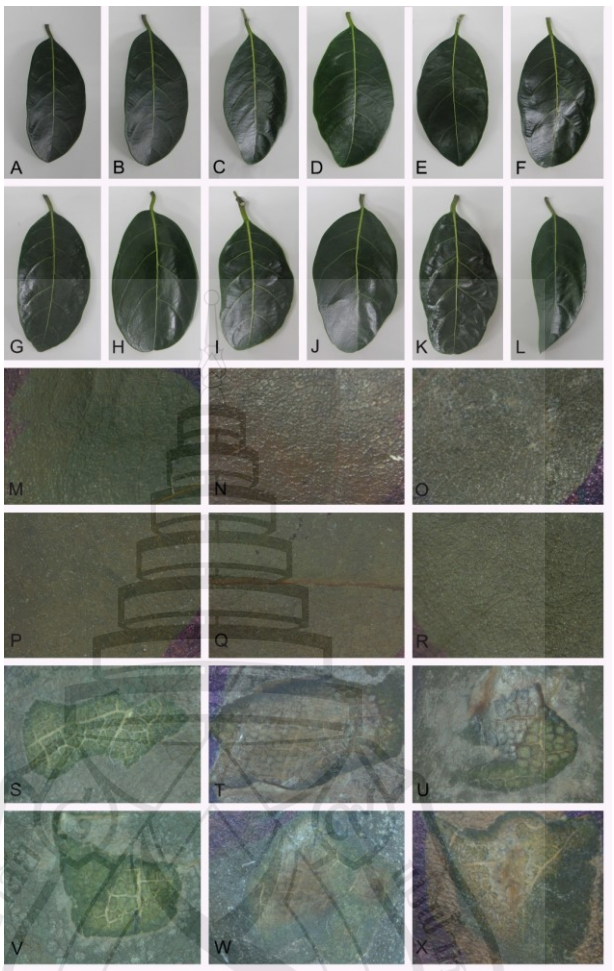


Note Results of the pairwise homoplasy index (PHI) test of closely related species using both LogDet transformation and splits decomposition.

Figure 5.3 Results of the pairwise homoplasy index (PHI) test

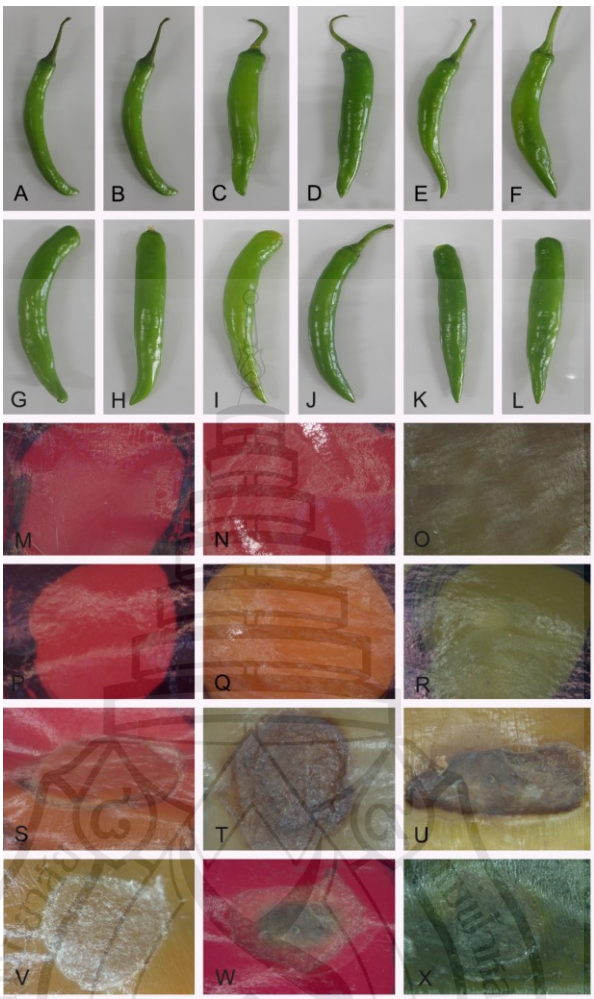
5.3.3 Pathogenicity Assay

The wounded samples inoculated with colonised mycelium plugs showed symptoms after 24 hours, while symptoms on the non-wounded samples took another 72 hours to appear. The wounded samples inoculated with conidial suspension showed symptoms 96 hours after initial inoculation and 48 hours earlier than non-wounded samples. The samples inoculated with the isolate formed orange to pale brown lesions. No symptoms developed on the negative controls. To confirm Koch's postulates, re-isolation was performed from the lesions formed on banana fruits. The isolates had morphological characters identical to that of MFLUCC 18–1167. Most of the inoculated samples showed extensive sign of necrosis after 9 days of initial inoculation.



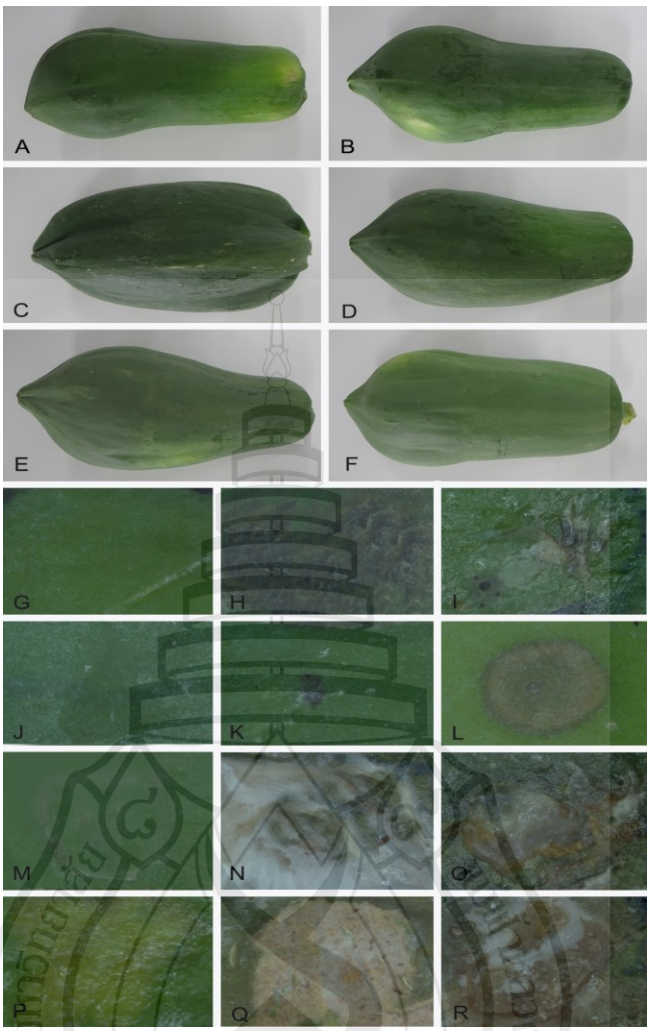
Note A-L *Artocarpus heterophyllus* leaves selected for pathogenicity test. M, P Non-wounded negative control (conidial suspension and mycelium plug). N-O Non-wounded leaves inoculated with conidial suspension. Q-R Non-wounded leaves inoculated with colonised mycelium plug. S, V Wounded negative control (conidial suspension and mycelium plug). T-U Wounded leaves inoculated with conidial suspension showing sign of necrosis after 7 days. W-X Wounded leaves inoculated with colonised mycelium plug showing sign of necrosis after 7 days.

Figure 5.4 Pathogenicity assay results of *C. artocarpicola* on *Artocarpus heterophyllus*



Note A-L *Capsicum* sp. selected for pathogenicity test. M, P Non-wounded negative control (conidial suspension and mycelium plug). N-O Non-wounded *Capsicum* sp. inoculated with conidial suspension. Q-R Non-wounded *Capsicum* sp. inoculated with colonised mycelium plug. S, V Wounded negative control (conidial suspension and mycelium plug). T-U Wounded *Capsicum* sp. inoculated with conidial suspension showing sign of necrosis after 7 days. W-X Wounded *Capsicum* sp. inoculated with colonised mycelium plug showing sign of necrosis after 7 days.

Figure 5.5 Pathogenicity assay results of *C. artocarpicola* on *Capsicum* sp.



Note A-F *Carica papaya* selected for pathogenicity test. G, J Non-wounded negative control (conidial suspension and mycelium plug). H-I Non-wounded *Carica papaya* inoculated with conidial suspension. K-L Non-wounded *Carica papaya* inoculated with colonised mycelium plug. M, P Wounded negative control (conidial suspension and mycelium plug). N-O Wounded *Carica papaya* inoculated with conidial suspension showing sign of necrosis after 14 days. Q-R Wounded *Carica papaya* inoculated with colonised mycelium plug showing sign of necrosis after 14 days.

Figure 5.6 Pathogenicity assay results of *C. artocarpicola* on *Carica papaya*

5.4 Discussion

The gloeosporioides species complex is mainly characterised by species with cylindrical conidia with rounded ends tapering slightly towards the base [257]. Several *Colletotrichum* species have been associated with *Artocarpus* worldwide. *Colletotrichum artocarpi* which causes leaf spots on *Artocarpus* is characterised by the presence of hyaline, reniform, oval or ovoid shaped conidia [299] whereas *C. artocarpicola* has unicellular, often 1-septate conidia that are cylindrical and sometimes slightly ovoid with rounded ends conidia. Unfortunately, there is no DNA sequence data available for *C. artocarpi*. Three other *Colletotrichum* species (*C. artocarpi, C. gloeosporioides, C. siamense*) recorded on *Artocarpus* are associated with leaf spots whereas *C. artocarpicola* is a saprobe colonising roots. *Colletotrichum gloeosporioides* is characterised by greyish white cultures [300], which is similar to *C. artocarpicola*. *Colletotrichum gloeosporioides* var. *minor* has been synonymized under *C. fructicola* which was initially isolated from *Ficus edulis* [301]. The colony of *C. siamense* is white at first, becoming pale brownish to pinkish whereas it is off-white to grey in *C. artocarpicola.*

Colletotrichum species associated with *Artocarpus* have several overlapping similarities in their conidial features, however, the conidia of *C. artocarpicola* are often 1-septate when germinating. The size of appressoria of *C. artocarpicola* are significantly different compared to *C. siamense* and *C. fructicola*. *Colletotrichum artocarpicola* possess setae whereas *C. fructicola* and *C. siamense* lack setae. The identification of *Colletotrichum* species based on morphological characters such as conidial features, sexual state and cultural characteristics is difficult due to overlapping morphological characters and phenotypic variation among species under different environmental conditions [138].

Combined phylogenetic analyses provided good evidence that the isolate in the current study belongs to a new species. *Colletotrichum artocarpicola* is closely related to the clade consisting of *C. endophytica* strains isolated from mango leaves, chilli pepper and tea plants in China. Our GCPSR analyses also showed that *C. artocarpicola* is distinguished as a separate species by genealogical concordance.

Colletotrichum artocarpicola has >1.5% bp difference in GAPDH and ACT gene regions compared to *C. endophytica* (MFLUCC 13-0418). GAPDH has been suggested as an important phylogenetic marker for accurate identification of species within the gloeosporioides complex [76] and hence the percentage divergence in nucleotides recovered herein substantiates new species rank for *C. artocarpicola*. Our multigene phylogeny also reveals some interesting findings for *C. endophytica*. *Colletotrichum endophytica* YN32-6, YN32-2, HN37-6, HN37-2 and CAUG28 did not cluster with the type strain of *C. endophytica* (MFLUCC 13-0418). Based on the importance of the GAPDH gene region for species delimitation in *Colletotrichum*, the strains YN32-6, YN32-2 and CAUG28 have over 1.5% bp difference compared to *C. endophytica* which suggest the need for further investigations to better circumscribe those strains.

Colletotrichum artocarpicola was initially isolated as a saprobe from jack fruit roots but artificial inoculation of the leaves of *Artocarpus heterophyllus*, *Carica papaya* and *Capsicum* sp. has shown that *C. artocarpicola* can be a pathogen, thus providing further evidence that species in gloeosporioides complex can be both saprobes and pathogens. Wang et al. [223] found that several species of gloeosporioides complex for example *C. endophytica* may show different life modes, switching between pathogenic and saprobic stage under different conditions.

CHAPTER 6

INTEGRATING DIFFERENT LINES OF EVIDENCE TO ESTABLISH A NOVEL ASCOMYCETE GENUS AND FAMILY (*ANASTOMITRABECULIA*, *ANASTOMITRABECULIACEAE*) IN *PLEOSPORALES*[5]

Abstract

A novel genus, *Anastomitrabeculia,* is introduced herein for a distinct species, *Anastomitrabeculia didymospora*, collected as a saprobe on dead bamboo culms from a freshwater stream in Thailand. *Anastomitrabeculia* is distinct in its trabeculate pseudoparaphyses and ascospores with longitudinally striate wall ornamentation. A new family, *Anastomitrabeculiaceae,* is introduced to accommodate *Anastomitrabeculia*. *Anastomitrabeculiaceae* forms an independent lineage basal to *Halojulellaceae* in *Pleosporales* and it is closely related to *Neohendersoniaceae* based on phylogenetic analyses of a combined LSU, SSU and *TEF1α* dataset. In addition, divergence time estimates provide further support for the establishment of *Anastomitrabeculiaceae.* The family diverged around 84 million years ago (MYA) during the Cretaceous period, which supports the establishment of the new family. The crown and stem age of *Anastomitrabeculiaceae* was also compared to morphologically similar pleosporalean families.[1]

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[5] This chapter was published as follows:

Bhunjun, C. S., Phukhamsakda, C., Jeewon, R., Promputtha, I. & Hyde, K. D. (2021). Integrating Different Lines of Evidence to Establish a Novel Ascomycete Genus and Family (*Anastomitrabeculia, Anastomitrabeculiaceae*) in *Pleosporales*. *Journal of Fungi, 7*, 94.

I declare that my contribution to this chapter was 95%.

Keywords: BEAST, *Dothideomycetes, Pleosporales, Poaceae,* Taxonomy, Three New Taxa, Trabeculate Pseudoparaphyses

6.1 Introduction

Pleosporales is the largest order within *Dothideomycetes* (*Ascomycota*) [302]. The taxonomic and phylogenetic relationships of families and genera within this order are well documented [60, 93, 302]. *Pleosporales* comprises two suborders, *Massarineae* and *Pleosporineae* [302]. *Pleosporineae* includes economically important plant pathogens and *Massarineae* includes mainly saprobes from terrestrial or aquatic environments [302]. Zhang et al. [302] revised 174 genera and accepted 26 families in *Pleosporales*. The suborder *Massarineae* was resurrected to accommodate five families, the *Lentitheciaceae*, *Massarinaceae*, *Montagnulaceae* (*Didymosphaeriaceae*), *Morosphaeriaceae* and *Trematosphaeriaceae* [302]. Hyde et al. [60] correlated morphology with phylogenetic evidence and accepted 41 families in this order. Tanaka et al. [267] introduced two new families, *Parabambusicolaceae* and *Sulcatisporaceae*, accepting 12 families in *Massarineae*. The family *Longipedicellataceae* was introduced, and the divergence time in *Pleosporales* was estimated with emphasis on *Massarineae* [303]. The crown age of *Pleosporales* was dated to 211 MYA and *Massarineae* was dated to 130 MYA [303]. Species boundaries in *Cucurbitariaceae* were revised [304] and the family, *Lentimurisporaceae*, was introduced in *Pleosporales* [305].

Species in this order are abundant and occur in terrestrial, marine and freshwater habitats [306–307]. The species can be epiphytes, endophytes or parasites of living leaves or stems, hyperparasites on fungi or insects, lichenized, or saprobes of dead plant stems, leaves or bark [306–307]. Currently, about 400 genera in 64 families are known in *Pleosporales* [302, 306, 308], with numerous coelomycetous and hyphomycetous taxa as their asexual morphs [302, 309].

Several pleosporalean taxa are pathogens associated with a broad range of hosts including bamboo. Bamboo (*Poaceae*) comprises over 115 genera with around 1500 species [310], can be found in diverse climates [311], and are widely distributed in

various forest types in Thailand [312–313]. It has been estimated that around 1100 fungal species belonging to over 200 genera have been described or recorded worldwide on bamboo and most of these bamboo-associated fungi are ascomycetes [314–315].

Divergence time estimates using molecular clock methodologies have been widely used in fungal taxonomy [303, 305]. Several studies have applied molecular dating to provide additional evidence for higher taxa ranking in *Pleosporales* [303, 306, 316]. In this study, we introduce a novel bambusicolous species, *Anastomitrabeculia didymospora* within *Anastomitrabeculia*, which is accommodated in a new family, *Anastomitrabeculiaceae*, based on morphology, multi-loci phylogeny and divergence times estimates.

6.2 Material and Methods

6.2.1 Sample Collection, Isolation and Identification

Dead bamboo culms were collected from a freshwater stream from Krabi province, Thailand, in 2015. The samples were incubated in plastic boxes with sterile and moist tissue at 25–30 °C for 3 days. Pure fungal colonies were obtained using single-spore isolation [67]. Germinating spores were transferred aseptically to potato dextrose agar (PDA) and malt extract agar (MEA) (Difco™). The cultures were incubated at 25 °C with frequent observations. Fungal characters were observed using a stereo microscope (Zeiss SteREO Discovery v. 8) fitted with an Axio Cam ERc5S and a Leica DM2500 compound microscope attached with a Leica MC190 HD camera. All microscopic measurements were carried out using Tarosoft (R) Image Frame Work program and the images were processed with Adobe Photoshop CS6 version 13.0 software (Adobe Systems, San Jose, CA, USA). The type specimens were deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand, and pure cultures were deposited at the Mae Fah Luang University Culture Collection (MFLUCC). The new taxon was linked with Facesoffungi numbers (FoF) [317] and Index Fungorum (Index Fungorum 2020, http://www.indexfungorum.org/, accessed on 2nd December 2020) and established based on guidelines recommended by Jeewon and Hyde [318].

6.2.2 DNA Extraction, Amplification and Sequencing

DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis were carried out as detailed in Dissanayake et al. [319]. Total genomic DNA was extracted from fresh mycelium with a Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) (Hangzhou, P.R. China) following the manufacturer's protocol. The nuclear ribosomal large subunit 28S rRNA gene (*LSU*) [320], the nuclear ribosomal small subunit 18S rRNA gene (*SSU*) [321] and the translation elongation factor 1-alpha gene (*TEF1α*) were amplified using primers (*LSU*: LROR/LR5, *SSU*: NS1/NS4 and *TEF1α*: 983F/2218R).

Table 6.1 Estimated DNA sequences and GenBank numbers used for the phylogenetic analyses in this study. The ex-type strains are in bold and the new taxon introduced in this study is indicated in blue

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Acrocalymma aquatica	**MFLUCC 11-0208**	JX276952	JX276953	-
Acrocalymma fici	**CBS 317.76**	KP170712	-	-
Acrocalymma medicaginis	**CPC 24340**	KP170713	-	-
Acrocalymma medicaginis	CPC 24341	KP170714	-	-
Acrocalymma medicaginis	CPC 24345	KP170718	-	-
Acrocalymma pterocarpi	**MFLUCC 17-0926**	MK347949	MK347840	-
Aigialus grandis	BCC 20000	GU479775	GU479739	GU479839
Aigialus mangrovis	BCC 33563	GU479776	GU479741	GU479840
Aigialus parvus	BCC 18403	GU479778	GU479743	GU479842
Aigialus rhizophorae	BCC 33572	GU479780	GU479745	GU479844
Aliquandostipite khaoyaiensis	CBS 118232	GU301796	-	GU349048
Amniculicola immersa	**CBS 123083**	FJ795498	GU456295	GU456273
Amniculicola lignicola	**CBS 123094**	EF493861	EF493863	-

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Amniculicola parva	**CBS 123092**	GU301797	GU296134	GU349065
Amorosia littoralis	NN 6654	AM292055	AM292056	-
Anastomitrabeculia didymospora	**MFLUCC 16-0412**	MW412978	MW412977	MW411338
Anastomitrabeculia didymospora	**MFLUCC 16-0417**	MW413899	MW413898	MW411339
Angustimassarina populi	**MFLUCC 13-0034**	KP888642	KP899128	KR075164
Angustimassarina quercicola	**MFLUCC 14-0506**	KP888638	KP899124	KR075169
Anteaglonium abbreviatum	**ANM 925a**	GQ221877	-	-
Anteaglonium globosum	SMH 5283	GQ221911	-	GQ221919
Anteaglonium parvulum	MFLUCC 14-0821	KU922915	KU922916	-
Antealophiotrema brunneosporum	**CBS 123095**	LC194340	LC194298	LC194382
Aquasubmersa japonica	HHUF 30468	LC061586	LC061581	-
Aquasubmersa japonica	**HHUF 30469**	LC061587	LC061582	-
Aquasubmersa mircensis	**MFLUCC 11-0401**	JX276955	JX276956	-
Arthonia dispersa	UPSC 2583	AY571381	AY571379	-
Ascocratera manglicola	BCC 09270	GU479782	GU479747	GU479846
Ascocylindrica marina	MD6011	KT252905	KT252907	-
Ascocylindrica marina	MD6012	KT252906	-	-
Ascocylindrica marina	MF416	MK007123	MK007124	-
Bahusandhika indica	GUFCC 18001	KF460274	-	-
Bambusicola massarinia	**MFLUCC 11-0389**	JX442037	JX442041	-
Berkleasmium micronesicum	BCC 8141	DQ280272	DQ280268	-
Berkleasmium nigroapicale	BCC 8220	DQ280273	DQ280269	-
Bimuria novae-zelandiae	CBS 107.79	AY016356	AY016338	DQ471087
Botryosphaeria dothidea	**CBS 115476**	AY928047	EU673173	AY236898
Brevicollum hyalosporum	MAFF 243400	LC271239	LC271236	LC271245
Brevicollum hyalosporum	**MFLUCC 17-0071**	MG602200	MG602202	MG739516
Brevicollum hyalosporum	PUFNI 17628	MH918671	-	-
Brevicollum versicolor	HHUF 30591	LC271240	LC271237	LC271246
Capnodium salicinum	**CBS 131.34**	DQ678050	DQ677997	-
Cladosporium cladosporioides	CBS 170.54	DQ678057	DQ678004	-

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Clematidis italica	MFLUCC 15-0084	KU842381	KU842382	-
Corynespora cassiicola	CBS 100822	GU301808	GU296144	GU349052
Corynespora smithii	CABI 5649b	GU323201	-	GU349018
Crassiparies quadrisporus	HHUF 30590	LC271241	LC271238	LC271248
Crassiparies quadrisporus	**HHUF 30409**	LC100025	LC100017	-
Crassiperidium octosporum	**KT 2144**	LC373108	LC373084	LC373120
Crassiperidium octosporum	KT 2894	LC373109	LC373085	LC373121
Crassiperidium octosporum	KT 3008	LC373110	LC373086	LC373122
Crassiperidium octosporum	KT 3029	LC373111	LC373087	LC373123
Crassiperidium octosporum	KT 3046	LC373112	LC373088	LC373124
Crassiperidium octosporum	KT 3188	LC373113	LC373089	LC373125
Crassiperidium octosporum	KT 3468	LC373114	LC373090	LC373126
Crassiperidium octosporum	KT 3604	LC373115	LC373091	LC373127
Crassiperidium octosporum	KT 3605	LC373116	LC373092	LC373128
Crassiperidium octosporum	MM 9	LC373117	LC373093	LC373129
Crassiperidium quadrisporum	**KT 27981**	LC373118	LC373094	LC373130
Crassiperidium quadrisporum	KT 27982	LC373119	LC373095	LC373131
Cryptoclypeus oxysporus	**HHUF 30507**	LC194345	LC194303	LC194390
Cryptocoryneum akitaense	**MAFF 245365**	LC194348	LC194306	LC096136
Cryptocoryneum japonicum	**MAFF 245370**	LC194356	LC194314	LC096144
Cryptocoryneum longicondensatum	**MAFF 245374**	LC194360	LC194318	LC096148
Cyclothyriella rubronotata	**CBS 141486**	KX650544	KX650507	KX650519
Cyclothyriella rubronotata	CBS 121892	KX650541	-	KX650516
Cyclothyriella rubronotata	CBS 385.39	MH867543	-	-
Cyclothyriella rubronotata	CBS 419 85	GU301875	-	GU349002
Delitschia didyma	UME 31411	DQ384090	AF242264	-
Delitschia winteri	CBS 225.62	DQ678077	DQ678026	DQ677922
Dendrographa decolorans	Ertz 5003	AY548815	AY548809	-
Dendrographa leucophaea f. minor		AF279382	AF279381	-
Dendryphion europaeum	CPC 22943	KJ869203	-	-

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Dendryphion europaeum	**CPC 23231**	NG_059120	-	-
Dendryphion nanum	MFLUCC 16-0975	MG208132	-	MG207983
Didymosphaeria rubi-ulmifolii	**MFLUCC 14-0023**	KJ436586	KJ436588	-
Dissoconium aciculare	CBS 204.89	GU214419	GU214523	-
Ernakulamia cochinensis	PRC 3992	LT964670	-	-
Flavomyces fulophazii	CBS 135761	KP184040	KP184082	-
Fuscostagonospora cytisi	**MFLUCC 16-0622**	KY770978	KY770977	KY770979
Fuscostagonospora sasae	**CBS 139687**	AB807548	AB797258	-
Fusculina eucalyptorum	**CBS 145083**	MK047499	-	-
Gordonomyces mucovaginatus	**CBS 127273**	JN712552		
Halojulella avicenniae	JK 5326A	GU479790	GU479756	-
Halojulella avicenniae	BCC 20173	GU371822	GU371830	GU371815
Halojulella avicenniae	PUFD542	MK026757	MK026754	-
Halojulella avicenniae	BCC 18422	GU371823	GU371831	GU371816
Halojulella avicenniae	BCC28357	KC555567	KC555565	-
Halojulella avicenniae	GR00584	KC555568	KC555566	-
Halotthia posidoniae	**BBH 22481**	GU479786	GU479752	-
Helminthosporium aquaticum	**MFLUCC 15-0357**	KU697306	KU697310	-
Helminthosporium velutinum	MAFF 243854	AB807530	AB797240	-
Helminthosporium velutinum	MFLUCC 13-0243	KU697305	-	-
Helminthosporium velutinum	**MFLUCC 15-0423**	KU697304	-	-
Hermatomyces iriomotensis	**HHUF 30518**	LC194367	LC194325	LC194394
Hermatomyces tectonae	**MFLUCC 14-1140**	KU764695	KU712465	KU872757
Hermatomyces thailandica	**MFLUCC 14-1143**	KU764692	KU712468	KU872754
Hobus wogradensis	**TI**	KX650546	KX650508	KX650521
Hysterium angustatum	**CBS 236.34**	FJ161180	GU397359	FJ161096
Hysterium angustatum	MFLUCC 16-0623	MH535893	MH535885	MH535878
Jahnula seychellensis	**SS2113**	EF175665	EF175643	-
Latorua caligans	**CBS 576.65**	KR873266	-	-
Latorua grootfonteinensis	**CBS 369.72**	KR873267	-	-

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Lentimurispora urniformis	**MFLUCC 18-0497**	MH179144	MH179160	MH188055
Leptosphaeria doliolum	**CBS 505.75**	GQ387576	GQ387515	GU349069
Leptoxyphium cacuminum	**MFLUCC 10-0049**	JN832602	JN832587	-
Leucaenicola phraeana	**MFLUCC 18-0472**	MK348003	MK347892	-
Lignosphaeria fusispora	**MFLUCC 11-0377**	KP888646	-	-
Lignosphaeria thailandica	**MFLUCC 11-0376**	KP888645	-	-
Lindgomyces ingoldianus	**ATCC 200398**	AB521736	AB521719	-
Longiostiolum tectonae	**MFLUCC 12 0562**	KU764700	KU712459	-
Lophiotrema eburnoides	HHUF 30079	LC001707	LC001706	-
Lophiotrema nucula	**CBS 627.86**	GU301837	GU296167	GU349073
Macrodiplodiopsis desmazieri	**CBS 140062**	KR873272	-	-
Magnicamarosporium diospyricola	MFLUCC 16-0419	KY554212	KY554211	KY554209
Massarina eburnea	CBS 473.64	GU301840	GU296170	-
Massariosphaeria phaeospora	**CBS 611.86**	GU301843	GU296173	-
Mauritiana rhizophorae	BCC 28866	GU371824	GU371832	GU371817
Medicopsis romeroi	CBS 122784	EU754208	EU754109	KF015679
Medicopsis romeroi	**CBS 252.60**	EU754207	EU754108	KF015678
Medicopsis romeroi	CBS 132878	KF015622	KF015648	KF015682
Murispora rubicunda	IFRD 2017	FJ795507	GU456308	GU456289
Neoastrosphaeriella krabiensis	**MFLUCC 11-0025**	JN846729	JN846739	-
Neohendersonia kickxii	**CBS 112403**	KX820266	-	-
Neohendersonia kickxii	CBS 122938	KX820268	-	-
Neohendersonia kickxii	CBS 114276	KX820267	-	-
Neohendersonia kickxii	CPC 24865	KX820270	-	-
Neohendersonia kickxii	CBS 122941	KX820269	-	-
Neomassaria fabacearum	**MFLUCC 16-1875**	KX524145	KX524147	KX524149
Neomassaria formosana	**NTUCC 17-007**	MH714756	MH714759	MH714762
Neomassarina chromolaenae	**MFLUCC 17-1480**	MT214466	MT214419	MT235785
Neomassarina pandanicola	**MFLUCC 16-0270**	MG298945	-	MG298947
Neomassarina thailandica	**MFLUCC 10-0552**	KX672157	KX672160	KX672163

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	SSU	*TEF1α*
Neomassarina thailandica	MFLUCC 17-1432	MT214467	MT214420	MT235786
Neotorula aquatica	**MFLUCC 150342**	KU500576	KU500583	-
Neotorula submersa	**KUMCC 15-0280**	KX789217	-	-
Occultibambusa bambusae	**MFLUCC 13-0855**	KU863112	KU872116	-
Occultibambusa pustula	**MFLUCC 11-0502**	KU863115	KU872118	-
Ohleria modesta	MGC	KX650562	-	KX650533
Ohleria modesta	**CBS 141480**	KX650563	KX650513	KX650534
Paradictyoarthrinium diffractum	MFLUCC 13-0466	KP744498	KP753960	-
Paradictyoarthrinium diffractum	MFLUCC 12-0557	KP744497	-	-
Paradictyoarthrinium hydei	MFLUCC 13-0465	MG747497	-	-
Paradictyoarthrinium tectonicola	MFLUCC 13-0465	KP744500	KP753961	-
Paradictyoarthrinium tectonicola	**MFLUCC 12-0556**	KP744499	-	-
Periconia thailandica	**MFLUCC 17-0065**	KY753888	KY753889	-
Phaeoseptum aquaticum	**CBS 123113**	JN644072	-	-
Phaeoseptum terricola	MFLUCC 10-0102	MH105779	MH105780	MH105781
Phyllosticta capitalensis	CBS 226.77	KF206289	KF766300	-
Piedraia hortae	**CBS 480.64**	GU214466	-	-
Polyplosphaeria fusca	**CBS 125425**	AB524607	AB524466	AB524822
Preussia lignicola	**CBS 363.69**	DQ384098	DQ384087	-
Preussia lignicola	CBS 264.69	GU301872	GU296197	GU349027
Pseudoastrosphaeriella bambusae	**MFLUCC 11-0205**	KT955475	KT955455	KT955437
Pseudoastrosphaeriella longicolla	**MFLUCC 11-0171**	KT955476	KT955456	KT955438
Pseudoastrosphaeriella thailandensis	**MFLUCC 10-0553**	KT955477	KT955456	KT955439
Pseudolophiotrema elymicola	**HHUF 28984**	LC194381	LC194339	LC194418
Pseudomassariosphaeria bromicola	**MFLUCC 15-0031**	KT305994	KT305996	KT305999
Pseudotetraploa curviappendiculata	**CBS 125426**	AB524610	AB524469	AB524825
Quadricrura septentrionalis	CBS 125428	AB524617	AB524476	AB524832

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	**SSU**	***TEF1α***
Racodium rupestre	L346	EU048583	EU048575	-
Racodium rupestre	L424	EU048582	EU048577	-
Ramusculicola thailandica	**MFLUCC 13-0284**	KP888647	KP899131	KR075167
Rimora mangrovei	JK 5246A	GU301868	GU296193	
Roccella fuciformis	Tehler 8171	FJ638979	-	-
Rostriconidium aquaticum	**KUMCC 15-0297**	MG208144	-	MG207995
Rostriconidium aquaticum	MFLUCC 16-1113	MG208143	-	MG207994
Salsuginea ramicola	KT 2597.1	GU479800	GU479767	GU479861
Salsuginea ramicola	CBS 125781	MH877872	-	-
Scorias spongiosa	CBS 325.33	MH866910	GU214696	-
Seriascoma didymospora	MFLUCC 11-0179	KU863116	KU872119	-
Sigarispora arundinis	JCM 13550	AB618998	AB618679	LC001737
Sigarispora ravennica	**MFLUCC 14-0005**	KP698414	KP698415	-
Splanchnonema platani	CBS 222.37	KR909316	KR909318	KR909319
Sporidesmioides thailandica	KUMCC 16-0012	KX437758	KX437760	KX437767
Sporidesmioides thailandica	**MFLUCC 13-0840**	NG_059703	NG_061242	KX437766
Sporormia fimetaria	UPS:Dissing Gr.81.194	GQ203729	-	-
Sporormiella minima	**CBS 52450**	DQ468046	-	DQ468003
Stagonospora pseudocaricis	**CBS 135132**	KF251762	KF251259	KF252741
Stemphylium vesicarium	**CBS 191.86**	DQ247804	DQ247812	DQ471090
Stemphylium vesicarium	CBS 714.68	DQ678049	DQ767648	DQ677888
Sulcatispora acerina	**KT2982**	LC014610	LC014605	LC014615
Sulcosporium thailandicum	**MFLUCC 12-0004**	KT426563	KT426564	-
Teichospora quercus	**CBS 143396**	MH107966	-	MH108030
Tetraplosphaeria sasicola	**KT 563**	AB524631	AB524490	AB524838
Torula gaodangensis	**MFLUCC 17-0234**	NG_059827	NG_063641	-
Torula herbarum	CBS 111855	KF443386	KF443391	KF443403
Triplosphaeria maxima	**MAFF 239682**	AB524637	AB524496	-
Tubeufia chiangmaiensis	**MFLUCC 11-0514**	KF301538	KF301543	KF301557
Tubeufia javanica	**MFLUCC 12-0545**	KJ880036	KJ880035	KJ880037

Table 6.1 (continued)

Taxon	Strain number	GenBank accession numbers		
		LSU	**SSU**	***TEF1α***
Vargamyces aquaticus	CBS 639.63	KY853539	-	-
Vargamyces aquaticus	HKUCC 10830	DQ408575	-	-
Versicolorisporium triseptatum	**HHUF 28815**	AB330081	AB524501	-
Westerdykella dispersa	CBS 297.56	MH869191	-	-
Westerdykella ornata	**CBS 379.55**	GU301880	GU296208	GU349021
Xenomassariosphaeria rosae	**MFLUCC 15-0179**	MG829092	MG829192	-

Polymerase chain reaction (PCR) was performed using PCR mixtures containing 5–10 ng DNA, 1X PCR buffer, 0.8 units Taq polymerase, 0.3 μM of each primer, 0.2 mM dNTP and 1.5 mM $MgCl_2$. PCR conditions were set at an initial denaturation for 3 min at 94 °C, followed by 40 cycles of 45 s of denaturation at 94 °C, annealing for 50 s at 56 °C for *LSU*, *SSU* and 52 °C for *TEF1α* and extension for 1 min at 72 °C, with a final extension of 10 min at 72 °C. All the PCR products were visualised on 1% Agarose gels with added 6 μL of 4S green dyes, per each 100 mL. Successful PCR products were purified and sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, P.R. China). All sequences generated in this study were submitted to GenBank (Table 6.1) and the ITS region of *Anastomitrabeculia didymospora* was deposited with the accession number MW413900 (MFLUCC 16-0412) and MW413897 (MFLUCC 16-0417).

6.2.3 Phylogenetic Analysis

The sequence data were assembled using BioEdit v. 7.2.5 [322] and subjected to a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest matches with taxa in *Pleosporales*. Reference sequence data of this order and some representatives of other orders of *Dothideomycetes* were downloaded from previously published studies [302]. The sequences were automatically aligned using default settings in MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) [323]. A combined dataset of three gene regions (LSU, SSU and TEF1α) was prepared and manually adjusted using BioEdit and AliView [324]. Phylogenetic analyses of the combined

dataset were performed using maximum likelihood, maximum parsimony and Bayesian inference method. Maximum likelihood analyses (ML), including 1000 bootstrap pseudoreplicates, were performed at the CIPRES web portal [325] using RAxML v. 8.2.12. Maximum parsimony analysis was conduct-ed using PAUP v.4.0b 10 [191] with the heuristic search option and number of replications 1000 each. The Tree Length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were documented.

The best model for different genes partition was determined in JModelTest version 2.1.10 [268] for posterior probability (PP). The general time reversible (GTR) model with a discrete gamma distribution plus invariant site (GTR+I+G) substitution model was used for the combined dataset. Posterior probabilities [326] were estimated by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.2.6 [193]. Four simultaneous Markov chains were run for 10 million generations and trees were sampled every 1000th generation, thus resulting in 10,000 trees. The suitable burn-in phase was determined by inspecting traces in Tracer version 1.7 [332]. The first 10% of generated trees representing the burn-in phase of the analyses were discarded, while the remaining trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree. The phylograms were visualized with FigTree v1.4.0 program [193] and edited using Adobe Illustrator CS6 v15.0 (Adobe Systems, USA).

6.2.4 Fossil Calibration and Divergence Time Estimates

Divergence times were estimated with BEAST 2.6.2 [304] based on the methodology described in Phukhamsakda et al. [303]. The aligned sequence dataset (*LSU*, *SSU* and *TEF1α*) used for the phylogenetic analyses were loaded into BEAUTI 2.6.2 to prepare the XML file. Nucleotide substitution models were determined using JModelTest version 2.1.10. The GTR+I+G nucleotide substitution model was applied to *LSU* and *TEF1α* partitions. The symmetrical (SYM) model with a discrete gamma distribution plus invariant site (SYM+I+G) substitution model was applied to the SSU partition. The data partitions were set with unlinked substitution, linked clock model and linked tree. An uncorrelated relaxed clock model with lognormal distribution was used. The Yule speciation process, which assumes a constant rate of speciation divergence, was used as the tree prior [326]. The analysis was performed in BEAST

2.6.2 for 100 million generations, sampling every 1000 generations. The effective sample size (ESS) was analysed with Tracer version 1.7 to check that the values were greater than 200, as recommended by Drummond et al. [274]. The first 20% trees were discarded as the burn-in phase and the remaining trees were combined in LogCombiner 2.6.2. The maximum clade credibility was calculated in TreeAnnotator v 2.6.2. The phylograms were visualized with FigTree v.1.4.0 program.

To estimate the divergence time for Anastomitrabeculiaceae, the fossil *Metacapnodium succinum* (*Metacapnodiaceae*) was used to set the crown age of *Capnodiales* using a normal distribution, mean of 100 MYA, SD of 150 MYA, giving 95% credibility interval of 346 MYA [303, 328]. The fossil *Margaretbarromyces dictyosporus* was used to calibrate the crown age of *Aigialus* (*Aigialaceae*) using a gamma distribution, with an offset of 35 MYA, a shape of 1.0, scale of 25, providing 95% credibility interval of 110 MYA [303, 327–330]. The split between *Arthoniomycetes* (outgroup) and *Dothideomycetes* was used as the secondary calibration using a normal distribution, mean of 300 MYA, SD of 50 MYA, giving 95% credibility interval of 382 MYA [328].

6.3 Results

6.3.1 Phylogenetic Analyses

The combined gene alignment comprised 196 strains and 2800 characters (*LSU*: 860 characters, *SSU*: 1039 characters and *TEF1α*: 901 characters). Among the 2800 characters, there were 1492 conserved sites (53%), 364 variable sites (13%) and 944 parsimony informative sites (34%). The parsimony analysis of the data matrix yielded one most parsimonious tree out of 1000 (CI = 0.265, RI = 0.659, RC = 0.175, HI = 0.735, Tree Length = 7606). Based on BLAST search in the NCBI GenBank of the *LSU* gene, the newly generated taxon MFLUCC 16-0412 and MFLUCC 16-0417 show 95% similarity to *Crassiperidium quadrisporum* (KT 27981 and KT 27982). The topology of the phylogenetic tree based on the *LSU* gene was generally congruent with the overall topology of the tree based on the combined dataset. Phylogenetic trees generated from maximum likelihood, maximum parsimony and Bayesian analysis of the combined

dataset resulted in similar topologies with some exception. The position of *Cyclothyriellaceae* and *Longiostiolaceae* differed between the three methods. The best scoring RAxML tree had a final likelihood value of -40,523.297855 (Figure 6.1). The new taxon formed an independent lineage basal to the *Halojulellaceae* with strong Bayesian inference support and moderate support from maximum likelihood (0.99 PP/65% MLBT). A new genus *Anastomitrabeculia* is therefore introduced within *Anastomitrabeculiaceae* to accommodate the new species.

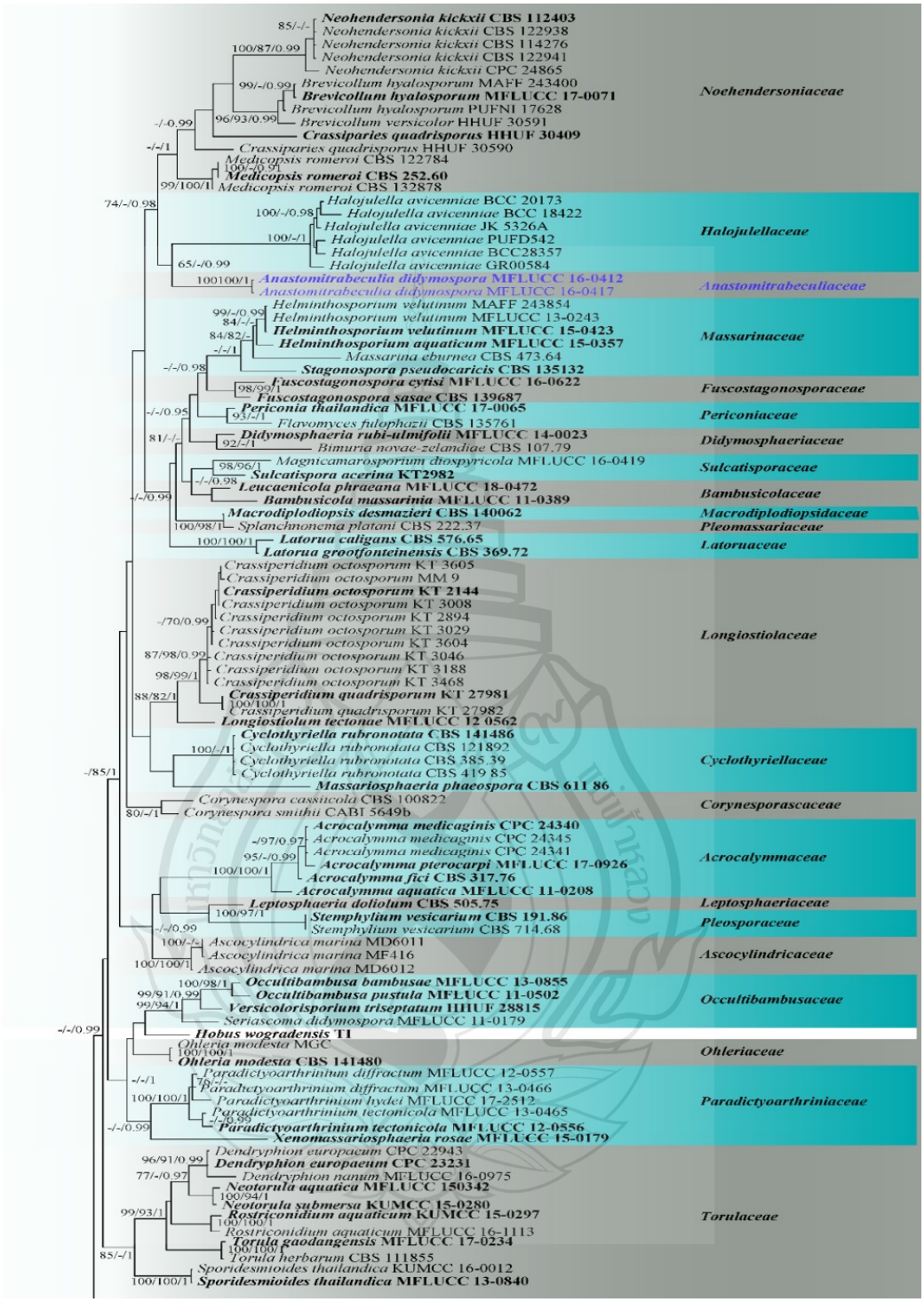
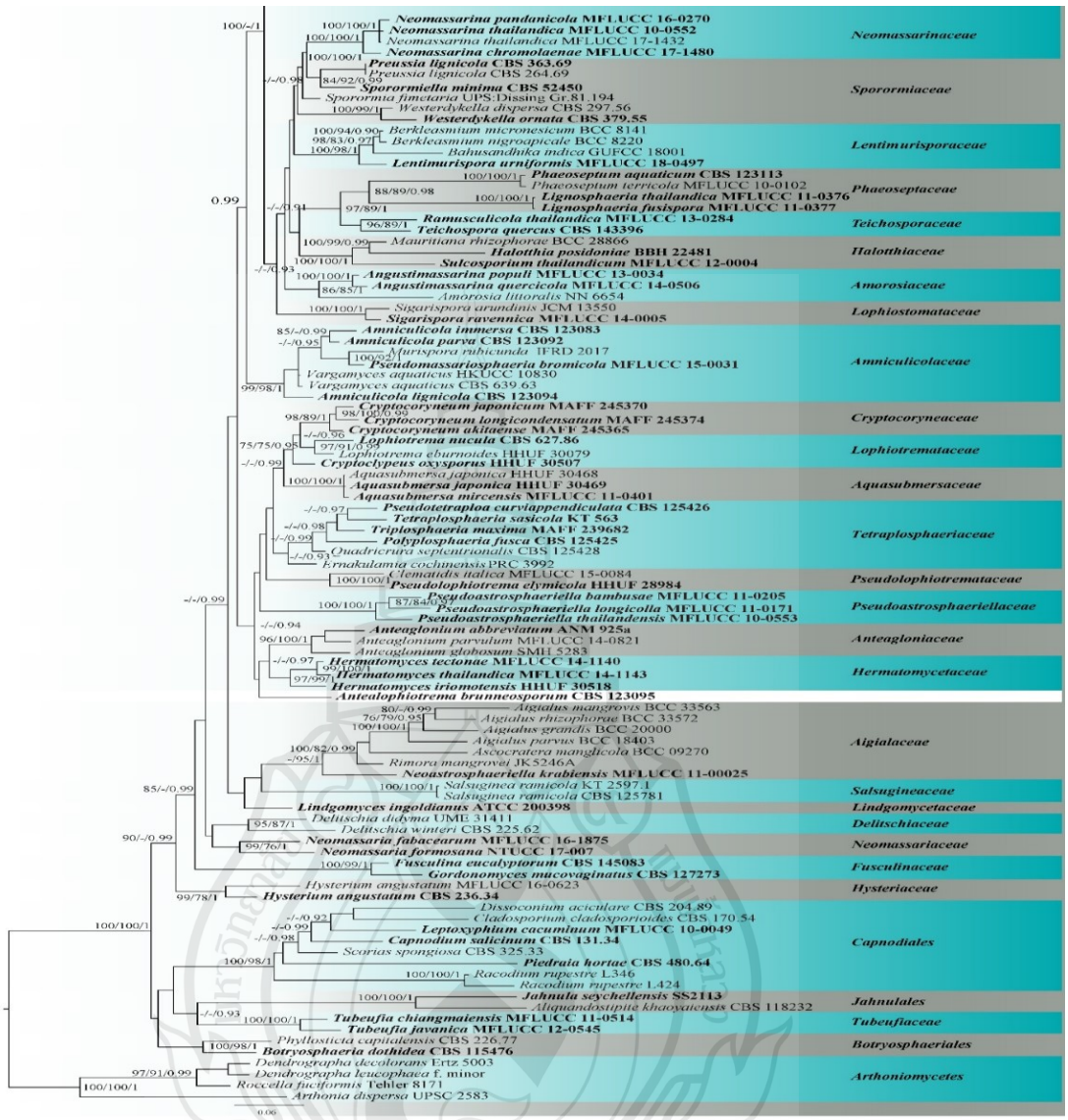


Figure 6.1 Phylogenetic analysis of the placement of related families in *Dothideomycetes* class



Note The best scoring RAxML tree based on a combined *LSU*, *SSU* and *TEF1α* dataset. RAxML bootstrap support and maximum parsimony values ≥60% (BT), as well as Bayesian posterior probabilities ≥0.90 (BYPP) are shown, respectively, near the nodes. The ex-type strains are in bold and the scale bar indicates 0.06 changes per site. The tree is rooted with species of *Arthoniomycetes* and the new taxon is the the new taxon is indicated in blue.

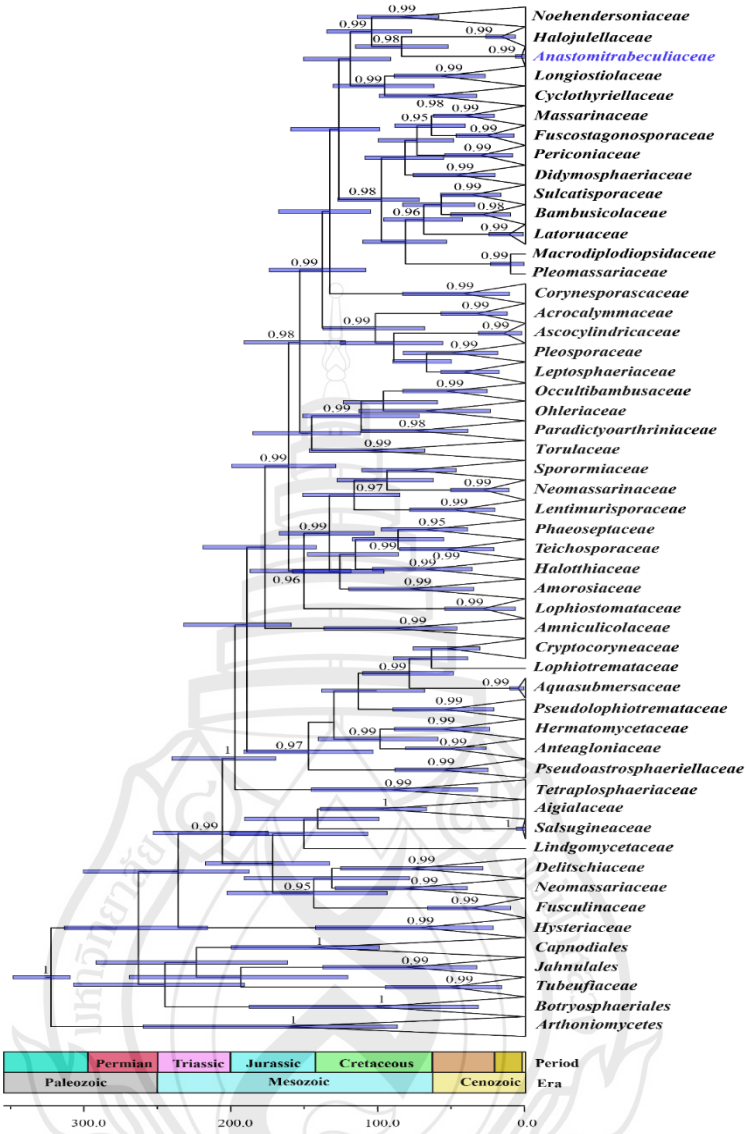
Figure 6.1 (continued)

6.3.2 Fossil Calibration and Divergence Time Estimates

The topology of the maximum clade credibility (MCC) tree (Figure 6.2) was congruent with the tree obtained from the Bayesian inference analysis and the maximum likelihood analysis. The crown age of *Dothideomycetes* is estimated at 263 MYA during the Permian period based on the MCC tree (Table 6.2). The split of *Arthoniomycetes* and *Dothideomycetes* occurred around 323 MYA during the Carboniferous period. The crown age of *Pleosporales* is estimated at 206 MYA, and *Hysteriales* diverged from *Pleosporales* approximately 236 MYA during the Triassic period. The crown age of *Anastomitrabeculiaceae* is estimated at around 2.6 MYA, and it diverged from *Halojulellaceae* at around 84 MYA. *Anastomitrabeculiaceae* formed an independent lineage with close relationship to *Halojulellaceae* with strong posterior probability in the MCC tree (0.99 BYPP). The divergence time of *Anastomitrabeculiaceae* was compared to Pleosporalean families with trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath (Table 6.3). The divergence time of *Anastomitrabeculiaceae* was also compared to *Didymosphaeriaceae* as they are morphologically similar by having trabeculate pseudoparaphyses and cylindrical asci.

Table 6.2 Divergence time estimates obtained from BEAST analysis. The median and the 95% Highest Posterior Density are provided in million years ago (MYA). The geological time scales are given based on the median node age

Nodes	Node Age	Geological Time Period
Arthoniomycetes–Dothideomycetes	323 (310–349)	Carboniferous
Dothideomycetes crown group	263 (216–313)	Permian
Hysteriales–Pleosporales	236 (188–300)	Triassic
Pleosporales crown group	206 (171–254)	Triassic
Capnodiales crown group	147 (99–200)	Jurassic
Anastomitrabeculiaceae stem group	84 (52–116)	Cretaceous
Aigialaceae–Aigialus sp.	37 (18–56)	Eocene
Anastomitrabeculiaceae crown group	2.6 (0.19–6.61)	Neogene



Note Maximum clade credibility (MCC) tree of families in *Dothideomycetes* using BEAST. Numbers at nodes indicate posterior probabilities (PP) for node support. Bars correspond to the 95% highest posterior density (HPD) intervals. Posterior probabilities greater than 0.95 are given near the nodes. The new taxon is indicated in blue. Geological time scales are given at the base together with scale in million years ago (MYA) [331].

Figure 6.2 Maximum clade credibility (MCC) tree of families in *Dothideomycetes* using BEAST

Table 6.3 Divergence time estimates obtained from BEAST analysis for families with similar morphology to *Anastomitrabeculiaceae*. The crown age and the stem age are provided in million years ago (MYA)

Families	Crown Age	Stem Age
Aigialaceae	102	141
Amniculicolaceae	90	177
Anastomitrabeculiaceae	2.6	84
Anteagloniaceae	52	98
Bambusicolaceae	29	57
Cyclothyriellaceae	66	95
Delitschiaceae	78	131
Didymosphaeriaceae	47	81
Fuscostagonosporaceae	26	63
Lindgomycetaceae	31	92
Neomassariaceae	82	131
Pseudoastrosphaeriellaceae	56	147
Tetraplosphaeriaceae	91	189

6.3.3 Taxonomy

Anastomitrabeculiaceae Bhunjun, Phukhams and K.D. Hyde, *fam. nov.*

Index Fungorum number: IF556817, Facesoffungi number: FoF 09521.

Etymology: Referring to the name of the type genus.

Saprobic on dead bamboo culms submerged in freshwater. Sexual morph: *Ascomata* immersed under a clypeus to semi-immersed, gregarious, uniloculate, globose to subglobose, carbonaceous, black. *Ostiole* central, apex well developed. *Peridium* multi-layered, sub-carbonaceous or coriaceous, with dark brown to hyaline cells arranged in a *textura angularis*. *Hamathecium* composed of numerous, filamentous, trabeculate pseudoparaphyses, septate, anastomosing between the asci and at the apex. *Asci* bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, bulbous pedicel, with an ocular chamber. *Ascospores* biseriate, broadly fusiform, septate, smooth-walled, with wall ornamentation, surrounded by mucilaginous sheath.

Note: *Anastomitrabeculiaceae* is introduced to include *Anastomitrabeculia*, which is reported as a saprobe on bamboo culms. *Anastomitrabeculiaceae* is characterised by semi-immersed, coriaceous or carbonaceous ascomata with septate, trabeculate pseudoparaphyses and hyaline ascospores with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. *Anastomitrabeculiaceae* formed a well-supported independent lineage closely related to *Halojulellaceae*, but *Halojulellaceae* differs by its cellular pseudoparaphyses and golden-brown ascospores. Type genus: *Anastomitrabeculia* Bhunjun, Phukhams and K. D. Hyde

Anastomitrabeculia Bhunjun, Phukhams. and K. D. Hyde, *gen*. *nov*.

Index Fungorum number: IF556560, Facesoffungi number: FoF 09522

Etymology: Referring to the trabeculate pseudoparaphyses anastomosing between the asci and at the apex.

Colonies on natural substrate umbonate at the centre, circular, black shiny dots are visible on the host surface. *Ascomata* on surface of the host, immersed under a clypeus, gregarious, uniloculate, subglobose, carbonaceous. *Ostiole* orange pigment near ostiole. *Peridium* comprising multilayers of brown to hyaline cells of *textura angularis*, inner layers composed of thin, hyaline cells. *Asci* 8–spored, bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, with a bulbous pedicellate, rounded at the apex, with an ocular chamber. *Ascospores* biseriate, broadly fusiform, tapering towards the ends, hyaline, with guttules in each cell, constricted at the septa, with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath.

Note: *Anastomitrabeculia* is established as a monotypic genus. It is characterised by the presence of carbonaceous ascomata, with orange pigment near ostiole and ascospores with longitudinally striate wall ornamentation. *Anastomitrabeculia* is morphologically similar to members of *Pleosporales* in having perithecioid ascomata, bitunicate asci and hyaline ascospores.

Type species: *Anastomitrabeculia didymospora* Bhunjun, Phukhams and K. D. Hyde

Anastomitrabeculia didymospora Bhunjun, Phukhams and K. D. Hyde, *sp*. *nov*.

Index Fungorum number: IF556559; Facesoffungi number: FoF 09523 Figure 3

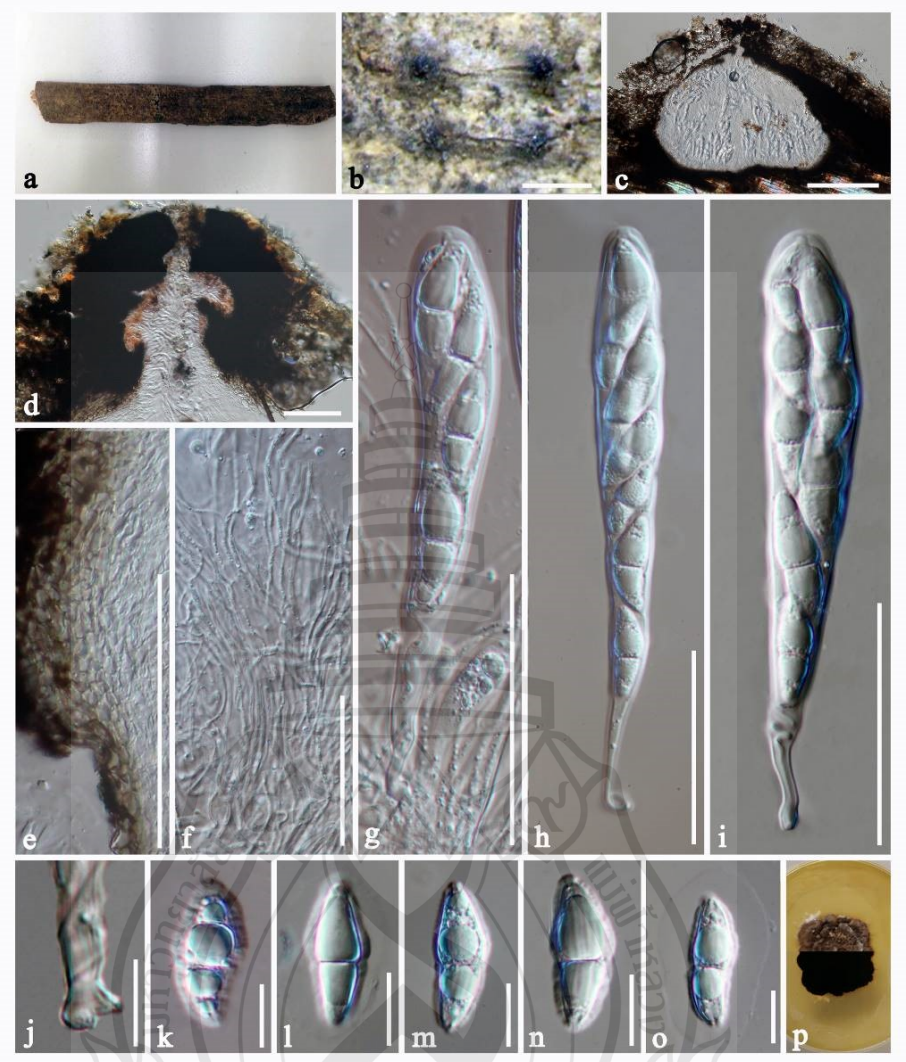
Etymology: Referring to the didymosporous ascospores.

Holotype: MFLU 20-0694

Saprobic on dead bamboo culms submerged in freshwater. Sexual morph: *Ascomata* 430–460 µm high, 435–575 µm diam., immersed under a clypeus to semi-immersed, gregarious, uniloculate, globose to subglobose, carbonaceous, rough, black, ostiolate. *Ostiole* 160 µm high, 270 µm diam., central, apex well developed, papillate, with pore-like opening, with periphyses filling the ostiolar canal, dark brown to black, orange pigment near ostiole. *Peridium* 6–18 µm wide, comprising 3–5 layers of brown to hyaline cells of *textura angularis*, inner layers composed of thin, hyaline cells. *Hamathecium* of dense, long, 0.8–1.25 µm wide ($\bar{x}$ = 1 µm, n = 50), filiform, filamentous, trabeculate pseudoparaphyses, septate, branched, embedded in a gelatinous matrix, anastomosing between the asci and at the apex. *Asci* 125–160 × 15–20 µm ($\bar{x}$ = 145 × 17 µm, n = 20), 8–spored, bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, with bulbous pedicellate, rounded at the apex, with an ocular chamber. *Ascospores* 18–28 × 7–10 µm ($\bar{x}$ = 22.5× 9 µm, n = 20), biseriate, broadly fusiform, tapering towards the ends, hyaline, 1-septate at the centre, constricted at the septum, cell above septate enlarged, straight, smooth-walled, with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. Asexual morph: Undetermined.

Culture characters: Ascospores germinating on MEA and PDA within 24 h with germ tubes developing from basal cells. Colonies on MEA and PDA umbonate at the centre, circular, friable, reaching 20 mm diameter after four weeks of incubation at 25 °C. Culture on MEA with white aerial mycelium, dark brown at the centre and paler towards the edge from above and below. Culture on PDA dark brown from above and below.

Material examined: THAILAND, Krabi province (8.1° N, 98.9° E), on dead bamboo culms, 15 December 2015, C. Phukhamsakda, KR001 (MFLU 20-0694, holotype), *ibid,* 18 December 2015 (MFLU 20-0695, paratype); ex-type living culture MFLUCC 16-0412; ex-paratype living culture, MFLUCC 16-0417.



Note *Anastomitrabeculia didymospora* (MFLU 20-0694, holotype). (a) Ascomata on bamboo. (b) Close-up of ascomata. (c) Vertical section of ascoma. (d) Ostiolar canal. (e) Peridium layer. (f) Trabeculate pseudoparaphyses. (g–i) Asci. (j) Pedicel. (k–o) Ascospores showing mucilaginous sheath. (p) Culture characteristics on PDA from above and below (9 cm diameter petri dish). Scale bar: (b) = 500 µm, (c) = 200 µm, (d–i) = 50 µm, (j–o) = 10 µm.

Figure 6.3 *Anastomitrabeculia didymospora* (MFLU 20-0694, holotype)

6.4 Discussion

In this study, we introduce a new species, genus and family for a collection of *Pleosporales* found on bamboo. The introduction of new taxa, even at the family level, is not surprising, considering that about 93% of fungi remain unknown to science despite ca. 2000 species described every year [1, 44]. Pleosporalean species can occur in terrestrial, marine and freshwater habitats [306–307]. Several studies have reported new pleosporalean taxa from freshwater or marine habitats or from bambusicolous hosts [267, 302]. *Pleosporales* have unique characters such as perithecioid ascomata typically with a papilla and bitunicate, generally fissitunicate asci, bearing mostly septate ascospores of different colours and shapes, with or without a gelatinous sheath [306]. The morphology of *Anastomitrabeculiaceae* is similar to members of the *Pleosporales* based on the presence of pseudoparaphyses, perithecioid ascomata, bitunicate asci and hyaline ascospores. *Anastomitrabeculiaceae* is characterised by semi-immersed to superficial ascomata, trabeculate pseudoparaphyses, cylindrical asci and ascospores with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. The newly discovered species formed a well-supported independent lineage basal to the *Halojulellaceae* based on phylogenetic analyses of the combined dataset (0.99 PP/65% MLBT). *Halojulellaceae* differs by its cellular pseudoparaphyses and golden brown ascospores [60]. The new taxon is also phylogenetically closely related to *Neohendersoniaceae*, which differs by its cellular pseudoparaphyses and smooth-walled ascospore [332]. A novel genus *Anastomitrabeculia* is therefore introduced to accommodate one new species, *Anastomitrabeculia didymospora.* A new family, *Anastomitrabeculiaceae*, is also introduced to accommodate this independent lineage.

Several pleosporalean families such as *Aigialaceae*, *Amniculicolaceae*, *Anteagloniaceae*, *Astrosphaeriellaceae*, *Bambusicolaceae*, *Biatriosporaceae*, *Caryosporaceae*, *Cyclothyriellaceae*, *Delitschiaceae*, *Didymosphaeriaceae*, *Fuscostagonosporaceae*, *Lindgomycetaceae*, *Melanommataceae*, *Neomassariaceae*, *Pseudoastrosphaeriellaceae*, *Striatiguttulaceae* and *Tetraplosphaeriaceae* share similar characters to *Anastomitrabeculiaceae* in having trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath [306]. The nature of pseudoparaphyses is

often overlooked, but they have taxonomic relevance at the genus and possibly family levels [306], but not at the ordinal level [332]. These families differ from *Anastomitrabeculiaceae* mainly by their ascospores, for example, *Aigialaceae* and *Amniculicolaceae* have brown and muriform ascospores [306]. *Anteagloniaceae* differs by having a peridium composed of dark brown cells of *textura epidermoidea,* cellular or trabeculate pseudoparaphyses and small, uniseriate ascospores [2]. *Astrosphaeriellaceae* differs by its brown, sub-fusiform to fusiform, obclavate to ellipsoidal, or limoniform ascospores [333] and *Biatriosporaceae* differs by its immersed ascomata and fusiform, dark brown ascospores [2]. *Caryosporaceae* differs by its broad-fusiform, ovoid or ellipsoid, brown ascospores [64]. *Bambusicolaceae* species have also been isolated from dead bamboo culms, but they differ from *Anastomitrabeculiaceae* by their cellular pseudoparaphyses and multi-seriate, smooth-walled ascospores [2]. *Cyclothyriellaceae* differs by its uniseriate, ellipsoid to fusiform, brown ascospores with several eusepta [65]. *Fuscostagonosporaceae* differs in having globose to subglobose ascomata, fissitunicate asci with long stipes and narrowly fusiform ascospores [66]. *Anastomitrabeculiaceae* shares several characters with *Didymosphaeriaceae* in having immersed ascomata formed under a clypeus, trabeculate pseudoparaphyses and cylindrical asci. *Didymosphaeriaceae* and *Melanommataceae* differ in having cellular or trabeculate pseudoparaphyses and brown, multi-septate, muriform ascospores [306]. *Lindgomycetaceae* differs by the presence of cellular or trabeculate pseudoparaphyses and brown, multi-septate ascospores with bipolar mucilaginous appendages [306]. *Neomassariaceae* differs by its immersed ascomata and ellipsoid ascospores. *Pseudoastrosphaeriellaceae* differs by its brown to reddish-brown ascospores with longitudinal ridges towards the ends and *Striatiguttulaceae* differs in having brown, ellipsoid ascospores with paler end cells. *Tetraplosphaeriaceae* differs by its immersed ascomata and slightly curved, pale brown ascospores [306].

Divergence time estimate has been widely used as supporting evidence to clarify taxonomic status of extant or novel families in fungal taxonomy [303, 305, 23–24, 26–27, 67]. In this study, the MCC tree was congruent with the topology of the trees generated from Bayesian inference analysis and maximum likelihood analyses. The divergence time estimates for the crown age of *Dothideomycetes* (263 MYA), the split of *Dothideomycetes* and *Arthoniomycetes* (323 MYA), the crown age of *Pleosporales*

(206 MYA) and the divergence of *Hysteriales* from *Pleosporales* (236 MYA) are similar to previous studies [11, 303, 306]. Hyde et al. [27] recommended that the divergence times of families should be between 50 and 150 MYA. The stem age is usually preferred to the crown age in taxa ranking as it is not affected by the sample size of the clade [27]. Based on the MCC tree, *Anastomitrabeculiaceae* and *Halojulellaceae* share the stem age of 84 MYA which supports the establishment of *Anastomitrabeculiaceae*.

The divergence time of *Anastomitrabeculiaceae* was also compared to Pleosporalean families with trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath (Table 6.3). *Cyclothyriellaceae* has an estimated crown age of 66 MYA and it diverged at 95 MYA. *Fuscostagonosporaceae* has a crown age of approximately 26 MYA and it diverged around 63 MYA. *Bambusicolaceae*, which was also isolated from dead bamboo culms, has a crown age of 29 MYA and a stem age of about 57 MYA. The stem age of *Anastomitrabeculiaceae* lies within the range of divergence times of those with similar morphology, but the crown age of *Anastomitrabeculiaceae* (2.6 MYA) is much earlier compared to these families. *Bambusicolaceae* was introduced by Hyde at al. [2] to include three bambusicolous taxa, and it currently has 15 species [306]. *Fuscostagonosporaceae* was introduced by Hyde at al. [66] to accommodate one bambusicolous taxon and it currently has four species [306]. Ariyawansa et al. [64] introduced the pleosporalean family, *Caryosporaceae*, which is morphologically similar to *Astrosphaeriellaceae* and *Trematosphaeriaceae* [306]. Based on Liu et al. [11], the stem age of *Caryosporaceae* (85 MYA) is similar to *Trematosphaeriaceae* (88 MYA) compared to *Astrosphaeriellaceae* (113 MYA), but the crown age of *Caryosporaceae* (2 MYA) is much earlier compared to *Astrosphaeriellaceae* (55 MYA) and *Trematosphaeriaceae* (65 MYA). *Astrosphaeriellaceae* currently has 111 species, and *Trematosphaeriaceae* has 103 species, whereas *Caryosporaceae* has ten species [306]. Compared to their morphologically similar families, the early crown of *Anastomitrabeculiaceae* and *Caryosporaceae* could be due to their smaller sample size. Therefore, further collections are needed for an accurate estimation of the crown age as it is affected by the sample size of the clade [328]. This could also be due to rapid speciation of pleosporalean fungal species given their high adaptation capabilities.

The estimated crown age of *Pleosporales* (206 MYA) lies within the early Triassic period. The origin of monocotyledons is estimated within the late Cretaceous period (around 145 MYA) [329]. This period is associated with the diversification of pleosporalean families, which continued during the early Cretaceous period when there was a major diversification and radiation of angiosperms, which favoured further diversification of Pleosporalean families to adapt to various hosts [56].

Hosts and their symbionts can speciate in parallel, which relates to a high level of congruence between the phylogeny of the hosts and their symbionts [330, 333]. Therefore, studies focusing on divergence time is important for a better understanding of host–pathogen interaction as well as co-evolutionary interactions [334]. This study uses a polyphasic approach based on morphology, multi-locus phylogenetic analyses and divergence time estimates. By implementing a polyphasic approach, we provide strong evidence for introducing the new family based on congruent results supporting the establishment of a new family.

CHAPTER 7

THE NUMBERS OF FUNGI: ARE THE MOST SPECIOSE GENERA TRULY DIVERSE?[6]

Abstract

Fungi play vital roles in the ecosystems as endophytes, pathogens and saprobes. The current estimate of fungal diversity is highly uncertain, ranging from 1.5 to 12 million, but only around 150,000 species have been named and classified to date. Since the introduction of DNA based methods, the number of newly described fungal taxa has increased from approximately 1,000 to around 2,000 yearly. This demonstrates the importance of DNA based methods to identify and distinguish species, especially morphologically similar taxa. Many novel species from recent studies have been found in historically understudied regions and habitats, but these still represent only a small percentage of the estimated species. In this paper, we introduce a new taxon in *Phyllosticta.* We also estimate the number of taxa in the top 30 most speciose genera as listed in Species Fungorum in GenBank. The genera are *Cercospora, Diaporthe, Meliola, Passalora, Phyllachora*, *Phyllosticta, Pseudocercospora, Ramularia* (ascomycetes), *Cortinarius*, *Entoloma*, *Inocybe*, *Marasmius*, *Psathyrella*, *Puccinia* and *Russula* (basidiomycetes). W e discuss why these genera have some of the largest number of species.[1]

[6] This chapter was published as follows:

Bhunjun, C. S., Niskanen, T., Suwannarach, N., Wannathes, N., Chen, Y.J., et al. & Hyde, K. D. (2021) The numbers of fungi: are the most speciose genera truly diverse? *Fungal diversity* (in press).

I declare that my contribution to this chapter was 95%.

Keywords: 1 New Taxon, *Ascomycota, Basidiomycota,* Fungal Diversity, Fungal Numbers, Host-specificity, Taxonomy

7.1 Introduction

Fungi are an ancient, diverse and heterogeneous group of organisms. They can be found in a wide range of habitats, ranging from the stratosphere [2] to the bottom of the Dead Sea [3], from the Antarctic glaciers [4] to torrid deserts [5] and from the gut of flies [6] to deep oceanic sediments [7]. Fungi and plants are intricately linked throughout the evolutionary history and it is hypothesized that plants may have never colonized land without fungi [335]. Fungi play key roles in ecosystems as decomposers, mutualists and pathogens, but the role of individual fungus in nature remains unknown [336]. As mutualists, they boost the host plant growth, fitness, stress tolerance and alter interactions with pests and pathogens [337]. As global environmental change continues to affect ecosystems and agricultural systems, these mutualists provide resilience and a better opportunity for plants to survive and adapt [1]. Fungi are essential for nutrient cycling as saprobes because of their ability to degrade cellulose, hemicellulose and lignin, thus releasing key plant nutrients back into the soil [338]. Without nutrient cycling, life on earth as we know it would not exist [1].

As pathogens, fungi can cause serious animal, human and plant diseases and the increasing number of infectious diseases caused by fungi is posing a growing threat to food security worldwide. There has been an unprecedented number of diseases caused by fungi which have resulted in severe die-offs and extinction of wild species. Several of these fungal pathogens were previously undescribed which highlight the importance of correctly estimating the number of species and identifying them to prevent disasters by fungal pathogens [339]. There has also been an increase in threats caused by emerging pathogens. For example, myrtle rust (*Austropuccinia psidii*) was only associated with plantations of introduced plants in South America until 2010 [340]. It was later detected in Australia with devastating effects on native forests and it has since been found in South Africa, New Caledonia and New Zealand

[340]. This is particularly worrying considering that myrtle rust successfully entered Australia and New Zealand undetected despite their exceptional vigilant biosecurity systems. Emerging pathogens can cause long-term devastation to ecosystems [337]. For example, Dutch elm disease (*Ophiostoma novo-ulmi*) and chestnut blight (*Cryphonectria parasitica*) have eliminated keystone tree species from ecosystems which has knock on effects for several organisms [141] .

Fungi play vital roles in several aspects of human life as food, medicine and as a source of income [44]. The phyla *Ascomycota* and *Basidiomycota* contain the majority of described fungal species with approximately 90,000 in *Ascomycota* and 50,000 in *Basidiomycota* [1]. The evolution of these two phyla occurred around 700 million years ago and they are capable of forming highly complex spore-bearing structures [1]. Ascomycetes have been used by humans for thousands of years to produce alcoholic drinks using yeasts [1]. Most medicines of fungal origin are from ascomycetes and this phylum also includes some of the most expensive food such as white truffle (*Tuber magnatum*) and the black truffle (*Tuber melanosporum*) [341]. Basidiomycetes are important in nutrient cycling and they also form an important part of human diet, for example button mushroom (*Agaricus bisporus*), the shiitake mushroom (*Lentinula edodes*) and chanterelle (*Cantharellus cibarius*) [1]. The global market for edible mushrooms is estimated at around 42 billion US dollars per year and these edible species are from 18 orders (114 genera) which represent a small fraction of fungal diversity [1]. Undescribed fungi could therefore represent massive potential at a time when there is a need to find new source of food, novel antibiotics, novel metabolites with biotechnological, industrial and pharmaceutical applications [8]. The discovery of undescribed fungi is important to reduce their risk of extinction by conserving them as extinction not only results in species loss, but also in loss of the unique evolutionary history which could represent irreplaceable features or functions [342].

7.1.1 Estimates of Fungal Diversity

Fungi are the second most species rich organism group [10]. The estimation of species number is vital for systematics, resources and classification [11]. To estimate diversity, one must establish what a species is. Species can be defined as genetic

clusters of organisms that are isolated from other clusters [343]. This isolation can be assessed with phenotypic and genetic data alike, but despite the simplicity of the definition, recognizing species is anything but simple. Different techniques have been used to estimate the global fungal species richness. Bisby and Ainsworth [344] estimated that there are 100,000 fungal species. Hawksworth [11] provided an estimation of 1.5 million fungal species based on the ratio of the number of vascular plants to fungi (1:6). This estimate was based on the assumption that that there are around 270,000 vascular plants and it has been most widely accepted estimate for two decades. Analysis of environmental DNA samples from soil community revealed an estimate of 3.5 to 5.1 million species. Blackwell [12] estimated that there are 5.1 million species based on high-throughput methods. Hawksworth and Lücking [345] estimated 2.2 to 3.8 million species based on the rate new species being described, cryptic species, unexplored habitats and species from environmental DNA samples. The revised estimate of 2.2 to 3.8 million is based on an updated ratio of the number of vascular plants to fungi (1:9.8) and based on the accepted number of around 380,000 vascular plant species [1]. Wu et al. (2019) estimated 11.7 to 13.2 million species based on data from culture-dependent and independent studies of the same samples where the ratio of cultured fungal numbers to operational taxonomic units was determined as 1:8.8.

7.1.2 Species Discovery Overview

Fungi and plants are intricately connected and evolve in parallel through co-speciation or host shifts [346]. There are over 150,000 species that have been named and classified which represents 10% of the expected diversity based on the relative conserved estimate of 1.5 million. Over the last two decades, the description of new fungal species has increased from around 1,000 to over 2,000 per year [1]. The increase in fungal species can be attributed to the use of DNA-based techniques which has been vital to delimitate between morphologically similar species [1]. There are around 2,100 new plant species discovered every year in the past two decades [347]. This is similar to the number of new fungal species discovered every year. Despite having similar rate of new discoveries every year, it is estimated that over 50% of plant species have been discovered whereas around 90% of fungal species remain

unknown [347]. This is possibly supported based on the comparison of new taxa described at higher ranks between the two kingdoms. In 2019, there were three classes, 18 orders, 48 families and 214 fungal genera introduced compared to one family and 87 plant genera introduced [347]. But, the suggestion that the majority of plant species has been discovered is debatable based on the steady rate of new species described each year.

In 2019, 2,300 new vascular plants species and 2,096 new fungal species were registered. There were 1,508 species introduced in *Ascomycota* and 588 species in *Basidiomycota*. The highest number of new ascomycetes species were described in *Hypocreales* (177 species), *Pleosporales* (167 species), *Lecanorales* (93 species) and *Capnodiales* (85 species) (Table 7.1). The highest number of new basidiomycetes species were described in *Agaricales* (184 species), *Polyporales* (87 species), *Russulales* (66 species) and *Boletales* (46 species) (Table 7.1). China (20%), Thailand (7%), the United states of America (6%) and Australia (5%) were the countries with the highest fungal species discovery in 2019. In 2020, 2,090 new species of vascular plants and 2,848 new fungal species were registered. There were 2,006 species introduced in *Ascomycota* and 843 species in *Basidiomycota*. The highest number of new ascomycetes species were described in *Pleosporales* (331 species), *Orbiliales* (329 species), *Hypocreales* (185 species) and *Diaporthales* (84 species) (Table 7.1). The highest number of new basidiomycetes species were described in *Agaricales* (314 species), *Tremellales* (78 species), *Polyporales* (77 species) and *Thelephorales* (49 species) (Table 7.1). China (10%), Thailand (9%), Europe (8%) and the United States of America (3%) reported the highest fungal species discovery in 2020. At the current rate of discovery, it would take over 650 years to describe all the fungal species based on the conserved estimate of 1.5 million. This is problematic considering that species have an extinction rate of 0.01-1% (up to 5%) per decade [348].

Table 7.1 Orders with most new species published in 2019 and 2020

Order	Number of species in 2019	Order	Number of species in 2020
Agaricales	184	*Pleosporales*	331
Hypocreales	177	*Orbiliales*	329
Pleosporales	167	*Agaricales*	314
Lecanorales	93	*Hypocreales*	185
Polyporales	87	*Diaporthales*	84
Capnodiales	85	*Tremellales*	78
Russulales	66	*Polyporales*	77
Boletales	46	*Thelephorales*	49

7.2 Case Studies

In this section, we examine the top 30 most speciose genera as listed in Species Fungorum. We refer to speciose genera as species-rich phylogenetic groups and show that species discovery in these genera remain high. We introduce 1 new species and discuss the likelihood of further new species being discovered. We also estimate the number of new species in GenBank using the best marker to delineate species based on maximum likelihood. In cases where there is no single marker that can be used to accurately delineate species, maximum likelihood is performed using the ITS region and the most parsimony informative gene.

Ascomycota Caval.-Sm.

Dothideomycetes O.E. Erikss. & Winka

Botryosphaeriales C.L. Schoch, Crous & Shoemaker

For the latest treatment of *Botryosphaeriales,* we follow Hongsanan et al. [170, 306].

Phyllostictaceae Fr.

Phyllostictaceae (as *Phyllostictei*) was first proposed by Fries [349]. Schoch et al. [80] placed *Phyllosticta* in *Botryosphaeriaceae* (*Botryosphaeriales*) and Wikee et al. [350] reinstated *Phyllostictaceae* in *Botryosphaeriales* to accommodate *Phyllosticta.*

Phyllosticta Pers.

Phyllosticta is a geographically widespread genus that is typified by *P. cruenta* [350]. Most of the species are plant pathogens associated with leaf blotch, leaf blight and black spots on fruits of a broad range of hosts [351]. These pathogens cause serious damage to the host through reduced photosynthetic ability and premature leaf or fruit fall [351, 352]. *Phyllosticta* can cause diseases of significant economic importance such as *P. citricarpa* which is associated with citrus black spot and it is regarded as a quarantine pest in Europe and the USA [351]. Another significantly important pathogen is *P. musarum* which can cause banana freckle disease [353] . *Phyllosticta* species have been recorded as endophytes from a wide range of hosts such as *P. capitalensis.* Species have also been recorded as saprobes such as *P. carpogena* and *P. ericae* [351].

7.2.1 How Many Species are There in *Phyllosticta*?

There are 3,213 epithets listed under *Phyllosticta* in Index Fungorum [30] and 2,073 epithets listed in Species Fungorum [354]. This is much higher than the number of confirmed species based on molecular data as many of these epithets were identified based only on morphology or host association and have since been accommodated in several other genera [355]. It remains to be confirmed if *Phyllosticta* species are generalists or host-specific as this may depend on the particular species or their lifestyles [350]. As pathogens, some species are host genus or family specific, whereas some species are generalists as endophytes [350]. For example, *P. sphaeropsoidea* causes leaf blotch disease specific to horse chestnut in Europe and North America, whereas the endophyte *P. capitalensis* can be found on a wide range of hosts [356].

There are over 2,900 sequences of ITS region, 2,090 sequences of LSU, 455 sequences of *act*, 202 sequences of *gapdh,* 145 sequences of *rpb2* and 37 sequences of

tef listed as *Phyllosticta* on GenBank. We estimate that there are around 650 new species based on the ITS region and 40 new species based on *act* gene on GenBank. However, the ITS region is not accurate for species level identification in *Phyllosticta*, therefore there is likely to be a relatively lower number of new taxa currently in GenBank. In an unpublished study in Guizhou Province, China, Wu Shiping identified 12 potential species on 27 hosts families. However, he was unable to isolate most taxa found and several of the 107 isolates obtained proved to be novel species. As most studies on this genus have been confined to economic hosts, studies of other plants are likely to result in significant novelty. There is likely to be a large number of species that will be discovered as some species appear to be host-specific. For example, freckle of banana is caused by *P. cavendishii*, *P. maculata* and *P. musarum* species and this work was only carried out in Asia-Pacific region [357]. Therefore, we would expect the number of species to double or triple with extensive study of different habitats, poorly studied hosts and other countries.

7.2.2 Taxonomy and Phylogeny

Phyllosticta was introduced by Persoon [358]. With the end of dual nomenclature for fungi, the generic name *Phyllosticta*, was chosen over *Guignardia* as it is the oldest and commonly used name [350]. The generic circumscription of *Phyllosticta* was based on the presence of pycnidial conidiomata containing aseptate, hyaline conidia surrounded by a mucilaginous sheath with an apical appendage [359]. Several species lack the mucilaginous sheath and apical appendage including *P. colocasiicola*, *P. minima* and *P. sphaeropsoidea* [350]. The conidial appendages in some species were reported to have disappeared with time or vary in size when mounted in water. Species delimitation was previously based only on morphology which was confusing as *Phoma* and *Phyllosticta* were both recognized as pycnidial fungi forming unicellular, hyaline conidia [350].

The first monograph of *Phyllosticta* was by van der Aa [359] who described and illustrated 46 species. The genus was revised based on the generic circumscription whereby van der Aa and Vanev [355] accepted 141 species and reclassified over 2700 species in several genera including *Asteromella* and *Phoma*. There is a lack of reference strains for many species and several species were

introduced based on their host association. Therefore, neo- or epitypification is required to clarify their taxonomic placement [350]. Several studies have used few genes other than the ITS region which is problematic as the ITS region has limited resolution for species delimitation in this genus [351]. Wikee et al. [350] described 12 novel species and designated eight epitype or neotype specimens. Jayawardena et al. [46] accepted 190 species and Norphanphoun et al. [360] introduced six species complexes to accommodate 85 species based on multi-locus phylogenetic analyses (ITS, LSU, *tef*, *act* and *gapdh*). Species delimitation based on the generic circumscription is problematic due to overlapping morphological characters. Therefore, it is recommended to apply a polyphasic approach based on morphology and multi-locus phylogenetic analyses to accurately identify *Phyllosticta* species [361]. In this study, we introduce a new species, *Phyllosticta doitungensis*.

Phyllosticta doitungensis Bhunjun & K. D. Hyde, *sp. nov.*

Index Fungorum number: IF559262; Facesoffungi number: FoF 10581; Fig. 7.1

Etymology: The epithet refers to the type locality.

Holotype: MFLU 21-0175

Saprobic on decaying leaf of *Dasymaschalon obtusipetalum* Jing Wang, Chalermglin & R. M. K.Saunders. Sexual morph: not observed. Asexual morph on host and culture**:** *Conidiomata* pycnidial up to 100 μm diam, solitary, black, semi-immersed, globose. *Conidiomata* (on PDA) up to 600 μm diam, solitary or arranged in clusters of up to 3, black, globose to ampulliform, base ovoid, including colorless to opaque conidial masses. *Pycnidial wall* consisting of several layers, up to 30 μm thick, outer layer of dark brown *textura angularis*, inner wall of 1–2 layers of hyaline *textura angularis* cells. *Ostiole* single, central, up to 20 μm diam. *Conidiophores* reduced to conidiogenous cells, subcylindrical to ampulliform. *Conidiogenous cells* 6−8 × 2–4 μm, terminal, subcylindrical, hyaline, smooth, proliferating several times percurrently near apex. *Conidia* 10–13 × 7–9 μm ($\bar{x}$ = 11 × 8 μm, n = 20), solitary, hyaline, aseptate, thin and smooth walled, with a single large central guttulate, broadly ellipsoid to ovoid, tapering towards a bluntly rounded apex, forming appressoria within 2 days, apical mucoid appendage not observed.

Culture characteristics: On PDA, colonies flat, fast growing reaching 4 cm after 14 days, initially white with abundant mycelium, lobate margins, gradually becoming grey after 2–3 days with white hyphae around the margin, eventually turning black; reverse black after 14 days.

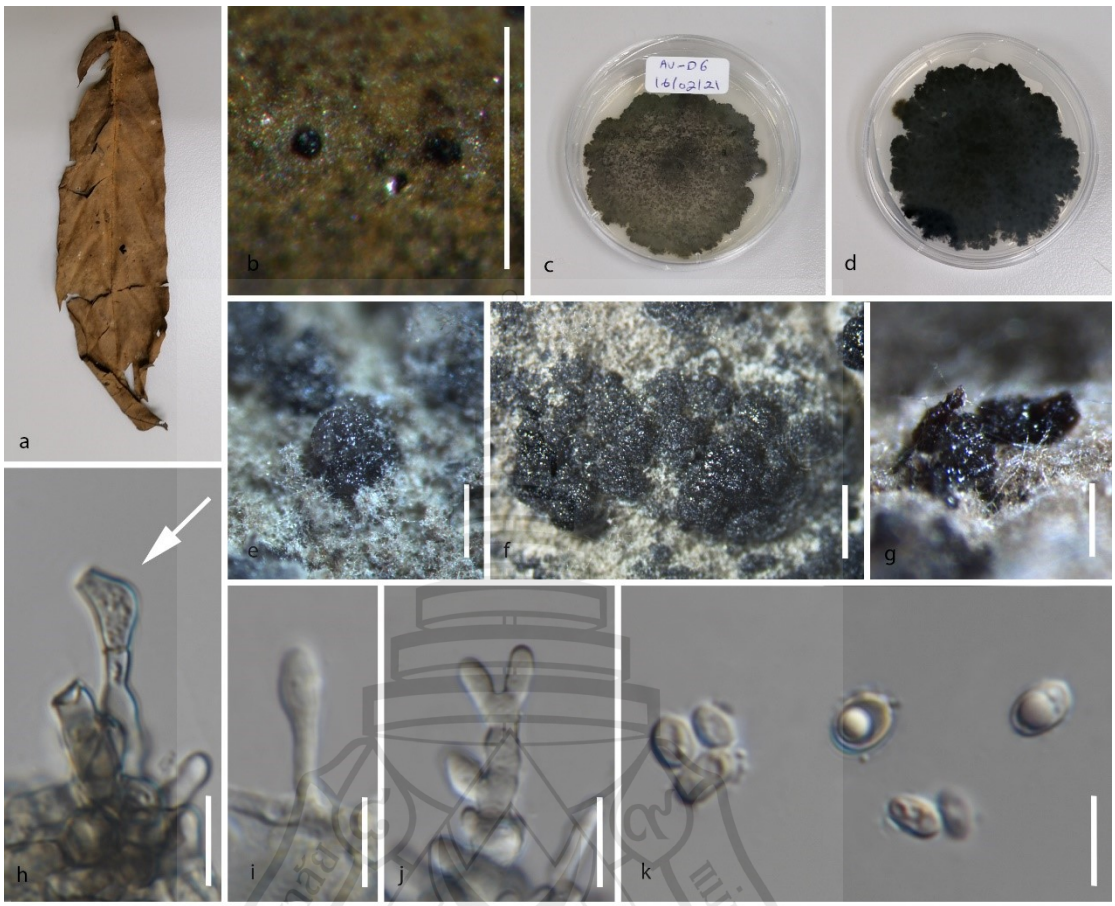
Habitat**:** on leaf of *Dasymaschalon obtusipetalum*.

Distribution**:** Known only from Thailand.

Material examined: THAILAND, Chiang Rai Province, Doi Tung, on leaf of *Dasymaschalon obtusipetalum*, 31 Jul 2020, CS Bhunjun, AV–D6 (MFLU 21-0175, holotype), ex-type living culture, MFLUCC 21-0074.

GenBank numbers: ITS: OK661033, LSU: OK661034, *tef*: OL345581*, rpb2*: OL345582.

Notes. *Phyllosticta doitungensis* formed a strongly supported sister clade to *P. capitalensis* (ML: 74%, BYPP: 0. 96, Figure 2). *Phyllosticta doitungensis* differs by 23 base pairs in the ITS region, 2 base pairs in LSU, 27 base pairs in *tef* and 42 base pairs in *rpb2* compared to *P. capitalensis* (CBS 128856). *Phyllosticta capitalensis* differs from *P. doitungensis* by having conidia which are enclosed in a mucilaginous sheath and bearing apical appendage [361]. Therefore, *P. doitungensis* is introduced as a novel species based on morphology and phylogenetic analyses. There are over 100 *Phyllosticta* species reported from Thailand, but this is the first report of *Phyllosticta* species on *Dasymaschalon obtusipetalum* [116]. This suggests that extensive sampling of understudied hosts is likely to reveal new species.



Note *Phyllosticta doitungensis* (MFLU 21-0175, holotype). a Host. b Appearance of conidiomata on the host leaf surface. c, d Colony on PDA 14 days after inoculation. e–g Formation of pycnidia on PDA. h–j Conidiogenous cells giving rise to conidia (h showing developing appressoria). k Conidia. Scale bars: b, e–g = 500 µm, h–k = 10 µm.

Figure 7.1 *Phyllosticta doitungensis* (MFLUXX, holotype)

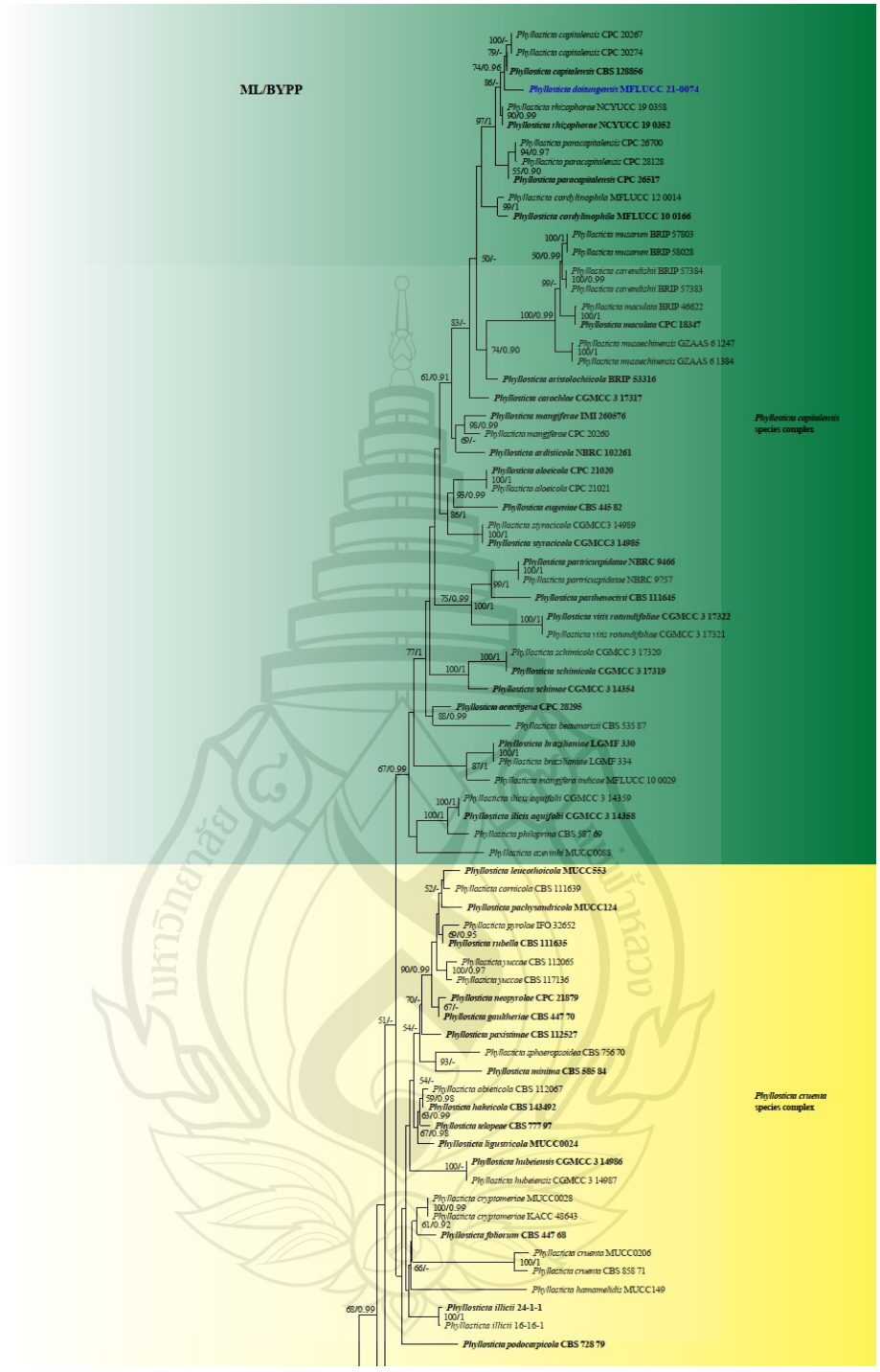
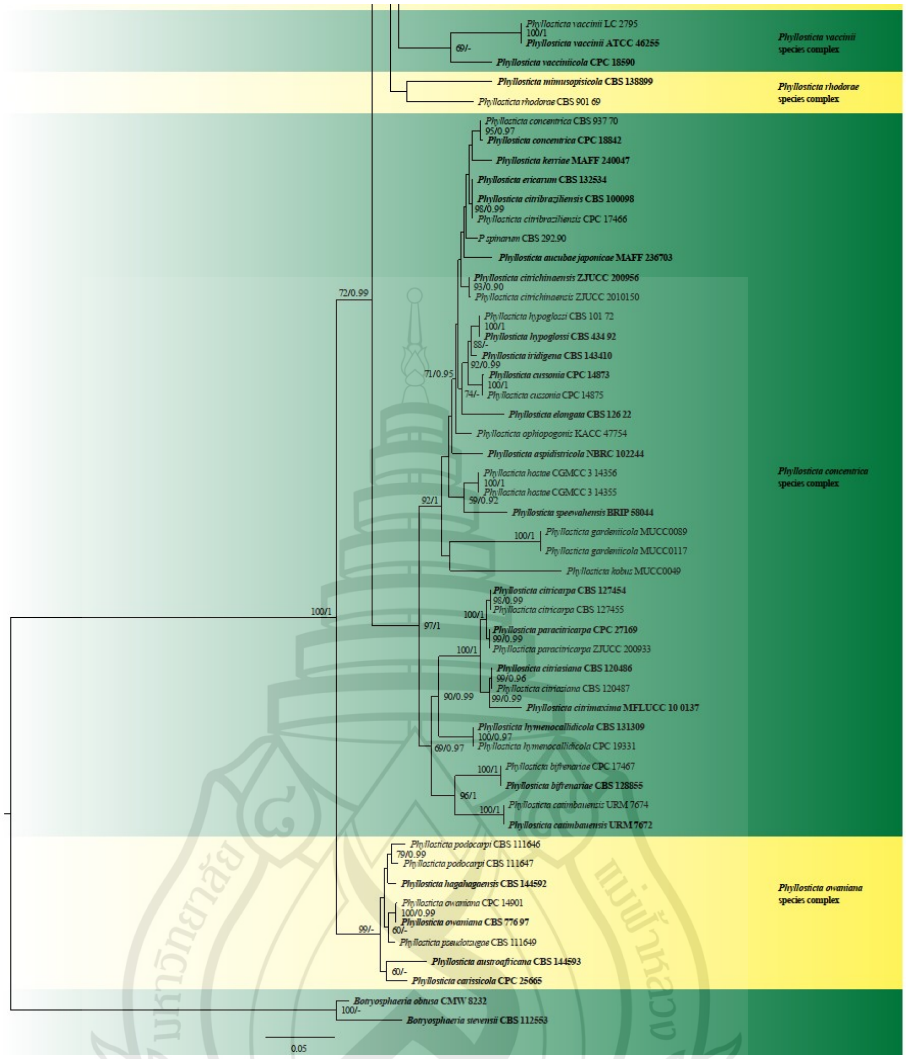


Figure 7.2 The best scoring RAxML tree for *Phyllosticta*



Note The best scoring RAxML tree based on a combined ITS, LSU, *tef, act, gapdh* and *rpb2* dataset. Related sequences were retrieved from GenBank and Norphanphoun et al. [360]. One hundred and twenty-six strains were included in the analysis of the combined loci which comprised 3127 characters after alignment. The tree is rooted with *Botryosphaeria obtusa* (CMW 8232) and *B. stevensii* (CBS 112553). The best scoring RAxML tree had a final likelihood value of -23828.412687. Hyphens (-) represent support values less than 50% BS/0.90 BYPP. The ex-type strains and reference strains are in bold and black. The newly generated sequence is in bold and blue.

Figure 7.2 (continued)

7.2.3 How Many New Species are there in the Other Selected Genera?

There are 3,159 epithets listed under *Cercospora* in Index Fungorum Index Fungorum [30] and 2,025 epithets listed in Species Fungorum [354]. There are 2,428 sequences of ITS region, 1,377 sequences of *his,* 1,347 sequences of *act*, 1,240 sequences of *tef*, 643 sequences of *cal*, 556 sequences of *tub*, 419 sequences of *gapdh* and 227 sequences of *rpb2* listed as *Cercospora* in GenBank. We estimate that there are around 117 new *Cercospora* species based on the ITS region and 36 new *Cercospora* species based on *gapdh* gene in GenBank. *Pseudocercospora* is a large genus comprising 1,705 epithets listed in Index Fungorum [30] and 1,642 epithets in Species Fungorum [354]. There are 1,990 sequences of ITS region, 1,201 sequences of *act* and 899 sequences of *tef* gene listed as *Pseudocercospora* in GenBank. We estimate that there are around 324 new *Pseudocercospora* species based on the ITS region and 189 new *Pseudocercospora* species based on *tef* gene in GenBank. There are 1,173 epithets listed under *Ramularia* in Index Fungorum [30] and 669 epithets listed in Species Fungorum [354]. There are 17,980 sequences designated as *Ramularia* species in GenBank with 973 sequences of ITS region, 588 sequences of *rpb2*, 410 sequences of *his*, 504 sequences of *act*, 480 sequences of *tef* and 454 sequences of *gapdh*. We estimate that there are around 210 new *Ramularia* species based on the ITS region and 115 new *Ramularia* species based on *his* gene in GenBank. There are 725 epithets listed under *Passalora* in Index Fungorum [30] and 677 epithets listed in Species Fungorum [354]. There are 2,180 sequences designated as *Passalora* species in GenBank. There are 254 sequences of LSU, 241 sequences of ITS region and 57 sequences of *rpb2* listed as *Passalora* in GenBank. We estimate that there are around 69 new *Passalora* species based on the ITS region and 83 new *Passalora* species based on the LSU gene in GenBank. There are more than 980 names listed under *Phomopsis* in Index Fungorum [30] and 884 epithets in Species Fungorum [354]. There are 1,142 epithets listed under *Diaporthe* in Index Fungorum [30] and 766 epithets in Species Fungorum [354]. There are 9,563 sequences of ITS region, 4,303 sequences of *tef*, 3,706 sequences of *tub* and 1,850 sequences of *cal* listed as *Diaporthe* in GenBank. We estimate that there are around 2,452 new *Diaporthe* species based on the ITS region and 189 new *Diaporthe* species based on *cal* gene in GenBank. There are 3,063 *Meliola* species listed in Index Fungorum [30]

and 2,629 species listed in Species Fungorum [354]. There are 45 sequences of LSU, 39 sequences of ITS region and 23 sequences of SSU listed as *Meliola* in GenBank. We estimate that there are 12 new *Meliola* species based on the LSU gene in GenBank. There are 1,514 epithets listed under *Phyllachora* in Index Fungorum [30] and 1,383 epithets in Species Fungorum [354]. There are 243 sequences of ITS region, 318 sequences of LSU and 219 sequences of SSU listed as *Phyllachora* in GenBank. We estimate that there are around 115 new *Phyllachora* species based on the ITS region and 196 new *Phyllachora* species based on LSU gene in GenBank.

There are 21,007 sequences designated as *Cortinarius* species in GenBank with 11,503 sequences of the ITS region. We estimate that there are around 1,168 new *Cortinarius* species based on the ITS region in GenBank. There are 2,503 names listed under *Entoloma* in Index Fungorum [30] and 2,239 names in Species Fungorum [354]. There are over 1,924 sequences of ITS region and 1,003 sequences of LSU listed as *Entoloma* in GenBank. We estimate that there are around 234 new *Entoloma* species based on the ITS region in GenBank. There are 2,142 names of *Inocybe* in the Index Fungorum [30] and 1,737 names in Species Fungorum [354]. There are 6,741 sequences of ITS region, 3,252 sequences of LSU and 1,068 sequences of *rpb2* listed as *Inocybe* in GenBank. We estimate that there are around 1,646 new *Inocybe* species based on the ITS region and 121 new *Inocybe* species based on the *rpb2* gene in GenBank. There are 1,993 epithets listed under *Marasmius* in Index Fungorum [30] and 1,531 epithets listed in Species Fungorum [354]. There are 3,379 sequences designated as *Marasmius* species in GenBank with 1,254 sequences of ITS region. We estimate that there are around 242 new *Marasmius* species based on the ITS region in GenBank. There are 1,062 *Psathyrella* names in the Index Fungorum [30] and 921 names in the Species Fungorum [354]. There are 1,296 sequences of ITS region and 340 sequences of LSU listed as *Psathyrella* in GenBank. We estimate that there are 222 new *Psathyrella* species based on the ITS region in GenBank. There are 2,946 epithets listed under *Russula* in Index Fungorum [30] and 2,390 epithets in Species Fungorum [354]. There are 10,862 sequences of ITS region, 5,809 sequences of LSU, 2,132 sequences of SSU, 1,227 sequences of *rpb2*, 636 sequences of *rpb1* and 648 sequences of *tef* listed as *Russula* in GenBank. We estimate that there are around 2,410 new *Russula* species based on the ITS region in GenBank. There are 20

names listed for *Picipes* in Index Fungorum [30] and Species Fungorum [354], but only 16 species have molecular data. There are 84 sequences of ITS region and 4 sequences of LSU listed as *Picipes* in GenBank. We estimate that there are around 13 new *Picipes* species based on the ITS region. There are 5,500 epithets listed under *Puccinia* in Index Fungorum [30] and 4,087 species listed in Species Fungorum [354]. There are over 2,750 sequences of LSU in GenBank and we estimate that there are around 130 new species based on the LSU gene in GenBank.

7.3 Discussion

Fungi are one of the most diverse groups in terms of shape, color, lifestyles and distribution throughout different ecosystems. The estimation of species number is vital for species conservation and ecosystems management as changes in fungal diversity can alter ecosystems functionality [362]. Determining the magnitude and patterns of fungal diversity remains a challenge for mycologists. Fungal species diversity estimates have differed significantly ranging from 1.5 to 12 million based on different approaches [13]. Despite increasing interest in correctly estimating the number of species, these studies do not represent reliable global estimates due to biases in the sampling size as these estimates were based on limited sampling which were extrapolated to provide a global diversity estimate [363–364]. For example, the estimated 1.5 million species were based on data only from the British Isles which had the highest ratio compared to other regions in the study. Several studies have investigated the ratio of fungal species to host which has varied between regions. For example, 3400 fungal species were discovered from 420 vascular plant species in a site in Surrey (United Kingdom), resulting in a fungal species to host ratio of 8:1 [345]. Metabarcoding approaches based on environmental samples have resulted in a fungal species to host ratio of 13:1 up to 19:1 [365]. This raises the question how realistic is the ratio of six fungal species to one host as the ratio has varied from 1.8:1 to 19:1 based on different approaches. Therefore, our knowledge of host specificity remains limited especially as new plant species continue to be discovered and several habitats remain unexplored. Another limitation of the estimated 1.5 million species is

that it was based on fungi recorded on plants, excluding fungi from several important habitats and other substrates such as insect fungi. Blackwell [12] estimate of 5.1 million species was based on the ITS region which is not suited to determine species diversity due to its limited informativeness at species level. Another limitation in species diversity estimates is taking into account species that are ubiquitous such as *Daldinia eschscholtzii* and *Exophiala alcalophila* [79, 366]. Therefore, future estimates should account for biogeographic distributions, levels of endemism and host specificity. It is also important to take extinction risk and diversity loss into account to accurately estimate global fungal species richness [364]. Fungal composition can be affected by several abiotic factors which poses a significant challenge for fungal conservation and management in a changing climate. There is currently the need to compile a database for the 150,000 described species with their geographic distribution and host association. This will provide a better understanding of fungal diversity and biogeography. Of the 150,000 formally recognized species, there are around 40,000 named fungal species in GenBank [345]. This highlights the lack of molecular data for formally recognized species. The high number of cryptic species represents another challenge and therefore the application of a polyphasic approach using different molecular approaches as well as morphology or ecology is important to accurately delinate species and to estimate species diversity [76, 367–368].

High throughput methods have revolutionized fungal ecology, recovering a larger diversity of fungi compared to culture-dependent approaches. These methods have discovered numerous new species and undescribed higher taxonomic ranks of fungi but the majority of these sequences cannot be assigned to any taxonomic rank [369]. There are over 100,000 species-level operational taxonomic units (OTUs) with no names and only a small amount of environmental sequences are deposited in GenBank [345]. A significant problem with using environmental sequence data to extrapolate global fungal species richness is the approach to define OTUs [345]. In theory, OTUs represent one physical fungal body in a microhabitat, but using the single barcoding locus ITS is not reliable to investigate fungal community [13]. Multiple loci are required for species delineation, but it is currently not possible to determine which environmental genes originated from which genome or cell, which makes it problematic to link the phylogeny of different genes [370]. There is a need to

develop classification systems based on environmental sequences as the incorporation of high throughput data into existing fungal classification systems would provide a comprehensive picture of global fungal diversity [1]. This could also result in the possibility to assign names to otherwise unknown OTUs [13]. Another issue that needs to be addressed in high throughput approach is in terms of reproducibility between environmental samples and protocols for naming species known only from DNA sequences [371]. These represent some of the challenges when estimating fungal diversity and shows the limitations of current estimates.

7.3.1 Fungal Species Discovery

Fungi and plants are intricately connected and new discoveries of species from the two kingdoms have been published at a rate of around 2,000 species per year [30]. There were however over 2,500 fungal species published in 2016 and 2020 whereas the rate of new plant species discovered remained constant at around 2100 [347]. The peak in the number of fungal species published could be attributed to several extensive studies that have introduced a large number of novel taxa. Hyde et al. [44] introduced two new families, 12 new genera, 82 new species, five new combinations and 25 new records from specimens that were collected mainly from Italy. Crous et al. [372] described six new genera, 70 new taxa and one new combinations from samples that were collected from many countries. Yuan et al. [373] introduced five new genera, 92 new species and eight new combinations based on materials collected from many countries. Mapook et al. [374] described one new family, 12 new genera, 47 new species, 12 new host records, three new taxonomic combinations associated with the invasive weed, *Chromolaena odorata* from northern Thailand. Phukhamsakda et al. [73] introduced two new families, 12 new genera, 50 new species, 26 new host records and ten new combinations from the cosmopolitan plant *Clematis* that were collected from Belgium, China, Italy, Thailand and the UK. Li et al. [375] identified two new orders, three new families, eight new genera, 107 new species and three new combinations in phyllosphere and soils samples mainly from China. These studies account for 18% of the species introduced in 2020.

7.3.2 Selected Genera in this Study

In this study, we have focused on the top 30 most speciose genera as listed in Species Fungorum [354]. All these genera have over 700 species listed on Index Fungorum [30] and over 650 species listed on Species Fungorum [354]. The most speciose ascomycete genera in this study is *Meliola* with over 2,600 epithets listed in Species Fungorum [354]. The most speciose basidiomycete genera selected is *Puccinia* with 5,500 species listed in Index Fungorum and over 4,000 species listed in Species Fungorum. The large number of species epithets in these genera is partly because species have been introduced based on host association or morphology, but these entries includes species that have been transferred to other genera and many are synonyms. This is reflected in the number of accepted species in these genera which is lower than the number of epithets listed in Species Fungorum. Despite the high number of species in these speciose genera, the number will increase with extensive study of different habitats, poorly studied hosts and other countries. The large number of species in these speciose genera could be partly attributed to their host specificity. The steady rate of new plant species discovered every year is also likely to result in a large number of fungal species. We therefore expect the number of species in all these genera to double or triple with extensive sampling.

7.3.3 Other Speciose Genera

Apiospora was recently separated from *Arthrinium* which is considered a speciose genus based on molecular data [312, 376]. *Apiospora* species can occur as endophytes, pathogens or saprobes and have been isolated from various substrates. Most collections of *Apiospora* species have been found on *Poaceae* as this is the most frequently sampled host [233]. Despite being found on a wide range of host and their ability to switch lifestyles, the number of *Apiospora* is low compared to other important pathogenic genera such as *Colletotrichum* which can also be found on a wide range of hosts and have been associated with different lifestyles [46, 114]. This suggest that a large number of species will be discovered in *Apiospora* with extensive sampling. *Picipes* was included in this study despite having only 20 species listed in Species Fungorum [354] as these species were previously classified as *Polyporus. Polyporus* has over 2400 epithets and it is the fourth most speciose basidiomycete

genera after *Agaricus* (4,621 epithets), *Cortinarius* (4,536 epithets) and *Puccinia* (4,087 epithets) in Species Fungorum [354]. *Picipes* species are abundant in tropical and subtropical regions. They play an important role in industrial biotechnology due to their ability to produce several enzymes as decomposers [377]. Most studies related to *Picipes* were confined to specific regions and therefore extensive sampling is likely to increase the number of species in this genus.

There are also several important genera that are speciose but do not occur in the top 30 in Species Fungorum [354]. *Colletotrichum* is an important plant pathogenic genus and was voted among the top ten fungal pathogens [81]. *Colletotrichum* species are endophytes, pathogens and saprobes [314]. It is one of the most common plant pathogens and virtually every crop grown throughout the world is susceptible to one or more species of *Colletotrichum* [378] . The taxonomy of species was previously based on morphology and culture characteristics [64]. This resulted in several taxa to be considered as synonyms as they represented a suite of morphologically indistinguishable taxa [70]. Molecular based approaches were used to delineate among the morphologically indistinguishable taxa, which resulted in the identification of 14 complexes and 248 accepted species in *Colletotrichum* [74]. There are 928 species epithets listed on Index Fungorum [30] and 501 species epithets listed on Species Fungorum [354] under *Colletotrichum*. This is much lower compared to the number of taxa recorded as *Colletotrichum* in GenBank (534,680 entries), which includes duplicate entries, host records and wrongly identified taxa, but could also represent several novel taxa. Most studies focusing on *Colletotrichum* have mainly focused on economically important or ornamental crops [74]. Several species lack molecular data or only have the ITS region which can resolve the placement of taxa up to the complex level [76]. As *Colletotrichum* species can be found on virtually every crop, the estimated number of species is expected to be close to the actual number of epithets listed on Index Fungorum with extensive sampling.

Penicillium is one of the most common fungi which can be found in a range of habitats, from soil to vegetation to air, indoor environments and various food products [379]. Despite being one of the most common fungi, they do not form part of the 30 most speciose genera. *Penicillium* main function in nature is the decomposition of organic materials, which has resulted in species being screened for the production of

novel enzymes [89]. *Penicillium* species have also been used in the food industry for example for the production of cheese and fermented sausages [380]. The species have been used for the production of penicillin, griseofulvin and the cholesterol-lowering drug mevastatin [379] . As endophytes, they protect the host against biotic stresses, to promote growth and protect the host against pathogens attack through the production of antagonist compounds [381]. *Penicillium* includes pathogens, allergens and mycotoxin-producing species [379]. The identification and classification of species were primary based on phenotypic and physiological characters [379]. Species identification based on morphology was problematic as differences in nutrients, temperature, lighting or humidity can alter the morphology of *Penicillium* species [379]. DNA-based approaches are important for species identification and for placing the strains into the species complex or one of the 25 sections [379]. There are 1,342 species epithets listed on Index Fungorum [30], 899 species epithets listed on Species Fungorum [354] and over 350 accepted species [379]. *Penicillium* species are ubiquitous fungi that can grow over a wide range of conditions and environments which suggest that they are likely to exponentially increase in number with extensive sampling of understudied habitats.

Pleurotus species (Oyster mushrooms) include several edible mushrooms that are known for their exceptional flavor and they can easily grow in various substrates [8]. They are predominantly found in tropical forests and they are among the most popular mushrooms worldwide [382]. *Pleurotus* species are primary decomposers that degrade many components of plant material including hemicellulose and lignin [383]. *Pleurotus* mushrooms are rich in proteins, dietary fiber, essential amino acids, carbohydrates, water-soluble vitamins and minerals, which makes them ideal as an alternative source of food [384]. *Pleurotus* species also have important medicinal properties such as anti-inflammatory, immunostimulatory, immunomodulatory and anticancer activity [384]. *Pleurotus ostreatus* has successfully been used to treat sites contaminated with pesticides and herbicides [385]. However, the demand for *Pleurotus* mushroom in the global market is lower than the button (*Agaricus bisporus*) and shiitake mushrooms (*Lentinula edodes*) [386]. This is mainly because they are highly perishable mushrooms with short self-life [387]. Species identification in this genus is difficult because it is largely based on morphology of the basidiomata [388].

Pleurotus species are classified in several complexes, but the morphology within these groups may be ambiguous because of environmental influences [388]. There are 757 species epithets listed on Index Fungorum [30], 571 species epithets listed on Species Fungorum [354], but only 25 accepted species of *Pleurotus* [389]. The low number of *Pleurotus* species could be due to their short self-life which makes finding them difficult, but it is expected to find several new *Pleurotus* species with extensive sampling in tropical forests.

In this paper, we have provided an insight of the top 30 genera as listed on Species Fungorum. Some of these genera are speciose partly because they are sampled and studied more often than others whereas some are speciose as they are of economic importance. These speciose genera have been found from a wide range of hosts and have different lifestyles while some are host specific, which could explain their high number of taxa. The discovery of new species continues to be high in regions that have been extensively studied, but there is also a need to sample unexplored habitats and substrates. These examples show that despite the high number of species in these genera, there is still much to be discovered.

CHAPTER 8

OVERALL CONCLUSIONS

This dissertation focused on the application of several different molecular-based methods to clarify species boundaries in two important pathogenic genera, *Bipolaris* and *Colletotrichum*. The species boundaries in these two genera have been revised in several studies, but they were mainly based only on morphology and phylogenetic analyses. This is the first study to implement several different approaches to provide a robust methodology for species delineation in these genera. The phylogenetic informativeness of different markers was also determined. This study also focused on the introduction of several novel taxa from dead plant material including the roots of *Artocarpus heterophyllus,* bamboo culms and leaf litter of *Dasymaschalon obtusipetalum*. The novelty of these taxa is supported by morphology and several molecular-based approaches including divergence times estimate, GCPSR and phylogenetic analyses. This is also the first study to provide guidelines for pathogenicity testing based on the implementation of modified Koch's postulates incorporating several criteria from Bradford Hill.

8.1 Clarification of Species Boundaries in *Bipolaris* and *Colletotrichum*

Fungi are an important group of organisms with potential uses in biotechnology, but they can also cause several serious human, animal and plant diseases. Therefore, it is important to accurately identify species based on a robust methodology. In this study, we revise the number of accepted species in two important plant pathogenic genera and provide guidelines on how to accurately delineate species in these genera.

Bipolaris species are important plant pathogens, but species delineation is problematic as the species are morphologically conserved. This is the first study to apply several approaches including phylogenetic analyses, ABGD and Objective clustering for species delineation in *Bipolaris* (Chapter 3). The *GAPDH* gene was determined the best single marker for species delimitation in *Bipolaris* which is in accordance with previous studies based only on phylogenetic analyses. We found that the majority of new host records that were named based on BLASTn searches of the ITS sequences could be erroneous. Forty-five species were accepted in *Bipolaris* and notes were provided for all the species including hosts and geographic distribution. Pairwise distance is often used to differentiate phylogenetically closely related taxa in several genera. However, in *Bipolaris*, the pairwise distances varied from 0 to 2.93 and 0 to 3.996 for closely related taxa based on the ITS and *GAPDH* datasets respectively. Therefore, we do not recommend the application of a specific threshold to delineate *Bipolaris* species. Based on findings from this study, we recommend performing phylogenetic analyses using multi-gene or the *GAPDH* gene for accurate species delineation in *Bipolaris*. We also recommend applying ABGD and Objective clustering to support the novelty of the taxon. GenBank is a comprehensive public database of nucleotide sequences and the number of novel taxa deposited as *Bipolaris* were estimated. There are at least 13 new *Bipolaris* species based on the ITS region and this suggests that there is a large number of novel species in GenBank compared to the number of accepted species.

Colletotrichum is one of the world's top 10 plant pathogenic genera and they have also been known to cause human infections. In this study, we elucidated species and species complex boundaries within *Colletotrichum* by using phylogenetic analyses, evolutionary analyses coupled with coalescent-based methods including mPTP and GMYC (Chapter 4). We also determined the phylogenetic importance of five different DNA barcodes for species delineation (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, ITS). The ITS marker effectively resolved the placement of most taxa up to complex level whereas the *GAPDH* and *TUB2* genes were the most informative markers for seven of the species complexes. *Colletotrichum axonopodi*, *C. parallelophorum* and *C. cariniferi* were accepted in the graminicola and dracaenophilum complexes respectively. We accepted 248 species which are mainly classified in 14 species

complexes and introduced a new species complex, the agaves complex with five closely related taxa. The caudatum and graminicola complexes were recovered as one based on all analyses and therefore, these two complexes should be treated as one, the graminicola-caudatum complex. This is the first study to estimate the divergence time for all the complexes in *Colletotrichum*, which varied from 4.8 MYA in the orchidearum complex to 32.2 MYA in the dematium complex. The evolutionary estimates from this study were congruent with divergence estimates from previous studies which were based on several calibration points, genome sequence data and a penalized likelihood method compared to the Bayesian analysis of five loci in our study. Achieving congruent results from different methodologies provided reliable support for divergence time estimation in our study. We recommend using phylogenetic analyses and coalescent-based methodologies based on a multi-locus dataset for accurate species delineation in *Colletotrichum* as there is currently no single marker that can discriminate between species in all the complexes.

8.2 Guidelines for Pathogenicity Testing

Plant diseases can be caused by multiple fungal genera that affect diverse hosts with different tissue specificities involving a myriad of symptoms. Fungal pathogens destroy one-third of all food crops annually and fungal diseases are responsible for the death of over 1.6 million humans every year. When identifying the cause of a plant disease, it is important to accurately identify the fungal species, as well as confirm if the organism is capable of causing damage to the host as several organisms can be present as endophytes or epiphytes on the plant surface. For accurate identification, different approaches based on a large taxon sampling and multi-gene analyses of mitochondrial, nuclear, ribosomal, and protein-coding genes should be used (Chapter 2). We recommend that novel pathogenic taxa should ideally be introduced with more than one strain to provide insights into intraspecific phenotypic diversity. We discussed the importance of Koch's postulates and the limitations related to pathogenicity testing of plants pathogens. We provide guidelines for pathogenicity testing based on Koch's postulates modified with aspects of Bradford Hill criteria

incorporating the biological gradient criterion, the consistency criterion, and the plausibility criterion. A set of protocols is provided as a guideline for pathogenicity testing of fungal pathogens on different parts of the plant along with a severity score guide. We recommend assessing disease severity using visual inspection and image analysis as the implementation of both methods will provide better accuracy and precision of severity measurement. It is also important to use statistical analyses to investigate sources of error in disease severity estimation from both methods.

8.3 Taxonomy and Phylogeny of Fungi Associated with Selected Hosts

This study has implemented a comprehensive approach to confidently introduce fungal taxa in several genera. This approach is based on the combination of morphology, phylogenetic analyses coupled with other molecular-based approaches. The genealogical concordance phylogenetic species recognition (GCPSR) approach is based on the idea that recombination within a lineage will create conflict between gene trees, with the transition from conflict to congruence representing the species limit. This approach was used to support the novelty of a new species, *Colletotrichum articarpicola* which was isolated from *Artocarpus heterophyllus* as a saprobe, but pathogenicity testing provided evidence that the new taxon can also be a pathogen (Chapter 5). Different lines of evidence were used to introduce a new genus *Anastomitrabeculia* and family, *Anastomitrabeculiaceae* from dead bamboo culms (Chapter 6). Phylogenetic analyses supported the placement of *Anastomitrabeculiaceae* in *Pleosporales* and its morphology is similar to members of the *Pleosporales* based on the presence of pseudoparaphyses, perithecioid ascomata, bitunicate asci and hyaline ascospores. Divergence time estimates using molecular clock methodologies were used to clarify taxonomic status of the novel taxon in *Pleosporales*. *Anastomitrabeculiaceae* diverged at around 84 Mya which supported its establishment as a novel family. In this study, a new species, *Phyllosticta doitungensis* was also identified from leaf litter specimens of *Dasymaschalon obtusipetalum* collected from Doi Tung, Thailand (Chapter 7).

8.4 Research Advantages

Accurate identification of fungal pathogens is vital as the scientific name links the knowledge concerning a species including the biology, host range, distribution, and potential risk of the pathogen, which are vital for effective control measures. This dissertation has focused on the application of several molecular-based approaches for accurate species identification. This study has provided a robust framework to determine species boundaries and these approaches can also be used to support the novelty of taxa. The implementation of these approaches in future studies will allow species to be delineated objectively and repeatedly. This is the first study to provide the divergence time estimate for all the complexes in *Colletotrichum*. This study has also provided evidence that the graminicola-caudatum complex is one species complex. This study has also determined the best single marker for species delineation in *Bipolaris* using different approaches. There is a vast amount of molecular data that is generated every year and this also results in the discovery of several new species. Therefore, there is a need to apply new approaches such as ABGD which are less computationally intensive compared to phylogenetic analyses. This study also provided an estimate of the number of novel taxa in several ascomycete and basidiomycete genera based on data in GenBank. There could be 13 new *Bipolaris* species (based on ITS region), 40 *Phyllosticta* species (*act* gene) and 36 *Cercospora* species (*gapdh* gene). Several of these genera are important pathogens and this data can be vital to prevent disasters by fungal pathogens. This data is also important as it gives insights into their host and geographic distribution which can be targeted for the discovery of novel taxa.

Koch's postulates are usually used to determine if an isolate is capable of causing host damage in plants. However, these postulates do not apply to all pathogens. The Bradford Hill criteria have been extensively used for inferring causation but was never applied to plant pathogen. This is the first study to provide guidelines for pathogenicity testing based on Koch's postulates modified with aspects of Bradford Hill criteria. This study provides guidelines concerning testing different parts of the plant along with a severity score guide, which takes into consideration the

depth of lesions. These guidelines will lead to a uniform approach to pathogenicity testing and allow inter-studies comparison. The importance of using a suitable sample size to generate accurate mean and reliable results is reinforced. This study also highlights the importance of statistical analyses to investigate sources of error in disease severity estimation. We also reiterate the use of the term *associated with the* "*disease* symptoms" *of "the host plant*" when introducing novel plant pathogenic fungal species without proving the taxon is the causal agent based on the guidelines.

This research has contributed one new family, one new genus, a new species complex and three novel taxa with illustration and molecular data. This study has contributed to the knowledge of saprobic fungal communities that can be found on these substrates. Several fungal taxa introduced in this study have only one strain despite regular collections of the same substrate in the same region spanning several months. This highlights the rarity of some fungal species in nature. This also shows the importance of using several molecular approaches as in some cases single strains are not strongly supported based only on phylogenetic analyses or it can be difficult to determine species boundaries.

8.5 Future Work

To the knowledge accomplished in this study, future research should be carried out based on several points including:

8.5.1 Several molecular-based approaches have been applied in this study, but these represent only a small number of approaches that can be used for species delineation. There is also a need to apply a robust framework to revise the species boundaries of several important pathogenic genera including *Diaporthe* and *Fusarium.*

8.5.2 Several pathogenic genera cannot be delineated based on a single gene. As a result, the introduction of novel taxa requires sequence data of several loci which is impractical in terms of time and cost. This highlights the importance of screening for novel markers that are scattered through the genome.

8.5.3 A great diversity of fungi has been discovered from tropical and warm-temperate areas. Despite extensive study, Thailand continues to be a hotspot for fungal biodiversity as a large number of new fungal species are discovered every year. Therefore, extensive studies of understudied hosts and habitats are likely to yield a large diversity of novel fungi.

8.5.4 This study has only focused on the application of culture-dependent method to study the diversity of fungi. Future studies can include culture-independent methods such as high throughput sequencing to study the diversity of fungi from these hosts and compare the diversity recovered from both methods.

8.5.5 Koch's postulates hold major importance in pathogenicity testing, but they cannot be applied to uncultured taxa. This is due to the absence of viable microorganisms with which to reproduce the disease to satisfy Koch's third postulate. Therefore, insights from whole-genome data are vital for a better understanding of how to target the dark taxa which could represent important pathogens.

8.6 Publications

As a result of this thesis during my PhD project, I have published six papers as first author in SCI journals and one book chapter. I have also co-authored 13 SCI publications.

List of publications

Publication as first author

1. Bhunjun, C. S., Jayawardena, R. S., Wei, D. P., Huanraluek, N., . . . Hyde, K. D. (2019). Multigene phylogenetic characterisation of *Colletotrichum artocarpicola* sp. nov. from *Artocarpus heterophyllus* in northern Thailand. *Phytotaxa*, *418*(3), 273–286.
2. Bhunjun, C. S., Dong, Y., Jayawardena, R. S., Jeewon, R., . . . Sheng, J. (2019). A polyphasic approach to delineate species in *Bipolaris*. *Fungal Diversity*, *102*, 225–256.
3. Bhunjun, C. S., Phukhamsakda, C., Jeewon, R., Promputtha, I., & Hyde, K. D. (2020). Integrating different lines of evidence to establish a novel Ascomycete genus and family (*Anastomitrabeculia*, *Anastomitrabeculiaceae*) in *Pleosporales*. *Journal of Fungi*, *7*(2), 94.
4. Bhunjun, C. S., Phukhamsakda, C., Jayawardena, R. S., Jeewon, R., . . . Hyde, K. D. (2021). Investigating species boundaries in *Colletotrichum*. *Fungal Diversity*, *107*, 107–127.
5. Bhunjun, C. S., Phukhamsakda, C., & Hyde, K. D. (2021). Structure and Development of Ascomata. *Encyclopedia of Mycology*, *1*, 255–262.
6. Bhunjun, C. S., Phillips, A. J. L., Jayawardena, R. S., Promputtha, I., & Hyde, K. D. (2021). Importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's postulates. *Pathogens*, *10*, 1096.
7. Bhunjun, C. S., Niskanen, T., Suwannarach, N., Wannathes, N., . . . Hyde, K. D. (2021). The numbers of fungi: are the most speciose genera truly diverse? *Fungal Diversity* (in press).

Co-author publications

1. Jayawardena, R. S., Hyde, K. D., McKenzie, E. H. C., Jeewon, R., . . . Wang, Y. (2019). One stop shop III: taxonomic update with molecular phylogeny for important phytopathogenic genera: 51-75 (2019). *Fungal Diversity*, *98*(1), 77–160.
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3. Hongsanan, S., Hyde, K. D., Phookamsak, R., Wanasinghe, D. N., . . . Xie, N. (2020). Refined families of Dothideomycetes: Dothideomycetidae and Pleosporomycetidae. *Mycosphere*, *11*, 1553–2107.
4. Phukhamsakda, C., McKenzie, E. H., Phillips, A. J. L., Jones, E. B. G., . . . Hyde, K. D. (2020). Microfungi associated with *Clematis* (Ranunculaceae) with an integrated approach to delimiting species boundaries. *Fungal Diversity*, *102*(1), 1–203.
5. Hyde, K. D., Jeewon, R., Chen, Y. J., Bhunjun, C. S., . . . Lumyong, S. (2020). The numbers of fungi: is the descriptive curve flattening? *Fungal Diversity*, *103*, 219–271.
6. Chen, Y. J., Jayawardena, R. S., Bhunjun, C. S., Harishchandra, D. L., & Hyde, K. D. (2020). *Pseudocercospora dypsidis* sp. nov. (Mycosphaerellaceae) on *Dypsis lutescens* leaves in Thailand. *Phytotaxa*, *474* (3), 218–234.
7. Jayawardena, R. S., Hyde, K. D., Chen, Y. J., Papp, V., . . . Wang, Y. (2020). One stop shop IV: taxonomic update with molecular phylogeny for important phytopathogenic genera: 76-100 (2020). *Fungal Diversity, 103*, 87–218.
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12. Jayawardena, R. S., Bhunjun, C. S., Hyde, K. D., Gentekaki, E., & Itthayakorn, P. (2021). *Colletotrichum*: lifestyles, biology, morpho-species, species complexes and accepted species. *Mycosphere, 12*(1), 519–669.
13. Jayawardena, R. S., Hyde, K. D., de Farias, A. R. G., Bhunjun, C. S., . . . Thines, M. (2021). What is a species in fungal plant pathogens? *Fungal diversity*, 1–28.

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APPENDIX

APPENDIX

ABSTRACT OF PUBLICATIONS

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One stop shop III: taxonomic update with molecular phylogeny for important phytopathogenic genera: 51–75 (2019)

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Abstract
This is a continuation of a series focused on providing a stable platform for the taxonomy of phytopathogenic fungi and organisms. This paper focuses on 25 phytopathogenic genera: *Alternaria*, *Capnodium*, *Chaetothyrina*, *Cytospora*, *Cyphellophora*, *Cytaria*, *Dactylonectria*, *Diplodia*, *Dothiorella*, *Entoleuca*, *Eutiarosporella*, *Fusarium*, *Ilyonectria*, *Lasiodiplodia*, *Macrophomina*, *Medeolaria*, *Neonectria*, *Neopestalotiopsis*, *Pestalotiopsis*, *Plasmopara*, *Pseudopestalotiopsis*, *Rosellinia*, *Sphaeropsis*, *Stagonosporopsis* and *Verticillium*. Each genus is provided with a taxonomic background, distribution, hosts, disease symptoms, and updated backbone trees. A new database (Onestopshopfungi) is established to enhance the current understanding of plant pathogenic genera among plant pathologists.

Keywords Classification · Database · Plant pathology · Phylogeny · Taxonomy · Symptoms · Systematics

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Multigene phylogenetic characterisation of *Colletotrichum artocarpicola sp. nov.* from *Artocarpus heterophyllus* in northern Thailand

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Abstract

A new species, *Colletotrichum artocarpicola*, on *Artocarpus heterophyllus* from Chiang Rai, Thailand, is introduced using both morphological and molecular approaches. Combined phylogenetic analysis of ITS, GAPDH, CHS-1, ACT and TUB2 sequence data demonstrate that *Colletotrichum artocarpicola* is a distinct species within the gloeosporioides species complex. The new species is illustrated and compared with related taxa, and evidence of its pathogenicity is provided.

Key words: 1 new species, molecular phylogeny, morphology, pathogenicity, saprobe

Introduction

The genus *Colletotrichum* was introduced by Corda (1831) and belongs to the family *Glomerellaceae* (Glomerellales, Sordariomycetes) (Maharachchikumbura *et al*. 2016, Hyde *et al*. 2019). Species of this genus can occur as endophytes and saprobes (Promputtha *et al*. 2007, Damm *et al*. 2009, Hyde *et al*. 2009a, b, 2014, Tao *et al*. 2013, Jayawardena *et al*. 2016 a,c), as well as plant and human pathogens (Than *et al*. 2008a, b, Yang *et al*. 2009, Phoulivong *et al*. 2010, Wikee *et al*. 2011, Hyde *et al*. 2014, Jayawardena *et al*. 2016a). It is worth noting that the same species can have different nutritional modes based on environmental conditions (Jayawardena *et al*. 2016a). For example, *C. fructicola* is a pathogen on various hosts (Weir *et al*. 2012) but can also be found as a saprobe and an endophyte (Vieira *et al*. 2014).

Species delimitation based on morphology alone is difficult due to overlapping morphological characters in asexual morphs and their sexual morphs are not often produced (Hyde *et al*. 2009a, Cannon *et al*. 2012). Misunderstanding of their host-specific nature has also led to considerable confusion concerning the species concept (Cannon *et al*. 2012, Jayawardena *et al*. 2016a). The application of a polyphasic approach including multi-loci sequence analyses, analyses of geographical, ecological and morphological data as suggested by Cai *et al*. (2009) is needed to provide a better taxonomic resolution for this genus. Index Fungorum (2019) lists 894 epithets (www.indexfungorum.org; accessed 5 August 2019) under the genus *Colletotrichum*, although there are fewer than 250 accepted species in 14 species complexes (Jayawardena *et al*. 2016b, Damm *et al*. 2018). We have been studying *Colletotrichum* in northern Thailand since 2007 and have identified 16 new species (Hyde *et al*. 2019, Samarakoon *et al*. 2018). Hyde *et al*. (2019) predicted that there may be more *Colletotrichum* species in northern Thailand and can be discovered through extensive sampling. *Artocarpus heterophyllus*, commonly known as jack fruit, is important commercially and is a delicacy in Thailand. Several species of *Colletotrichum* have been recorded from *Artocarpus* species in various parts of the world (Table 1).

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RESEARCH ARTICLE

MycoKeys

The genus *Simplicillium*

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Abstract

Simplicillium species have a wide host range and an extensive distribution. Some species are associated with rusts, as well as other plant pathogenic fungi and play an important role in biological control. In this study, two specimens of *Simplicillium* were collected from Chiang Mai Province, Thailand. *Simplicillium formicae* sp. nov. was isolated from an infected ant and *S. lanosoniveum* from *Ophiocordyceps unilateralis* which is a new host record. Species were initially identified using ITS gene sequences and confirmed using morphology coupled with phylogenetic analyses of a combined nrLSU, nrSSU, TEF and RPB1 dataset. *Simplicillium formicae* differs from other species in the genus by the presence of flask-shaped synnemata and phialides with intercalary nodes. *Simplicillium lanosoniveum* resembles other collections of the species by its completely solitary, tapering phialides and globose to ellipsoidal conidia which adhere in a slimly head. A key to species of *Simplicillium* is also provided.

Keywords

new species, Thailand, ant fungi, taxonomy, phylogeny

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A polyphasic approach to delineate species in *Bipolaris*

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Abstract
Bipolaris species are important plant pathogens with a worldwide distribution in tropical and temperate environments. Species recognition in *Bipolaris* has been problematic due to a lack of molecular data from ex-type cultures, the use of few gene regions for species resolution and overlapping morphological characters. In this study, we evaluate the efficiency of different DNA barcodes in species delimitation in *Bipolaris* by phylogenetic analyses, Automatic Barcode Gap Discovery and Objective Clustering. GAPDH is determined to be the best single marker for the genus. These approaches are used to clarify the taxonomic placement of all sequences currently named as *Bipolaris* in GenBank based on ITS and GAPDH gene sequence data. In checking various publications, we found that the majority of new host records of fungal species published in the Plant Disease journal from 2010 to 2019 were based on BLAST searches of the ITS sequences and up to 82% of those records could be erroneous. Therefore, relying on BLAST searches from GenBank to name species is not recommended. Editorial boards of journals and reviewers of new record papers should be aware of this problem. In naming *Bipolaris* species, whether new or known, it is recommended to perform phylogenetic analyses based on GAPDH using the correct taxon sampling for accurate results and the species relationship should have reliable statistical support. At least two new species are represented by molecular data in GenBank and we provide an updated taxonomic revision of *Bipolaris*. We accept 45 species in *Bipolaris* and notes are provided for all the species including hosts and geographic distribution.

Keywords Classification · DNA barcoding · Dothideomycetes · Integrative taxonomy · Pairwise differences · Pleosporales

Introduction

Bipolaris was introduced by Shoemaker (1959) with *B. maydis* as the type species and belongs to Dothideomycetes, Pleosporales, *Pleosporaceae* (Hyde et al. 2014, 2019; Wijayawardene et al. 2018; Phookamsak et al. 2019; Hongsanan

Kevin D. Hyde and Jun Sheng have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13225-020-00446-6) contains supplementary material, which is available to authorized users.

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Article
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Refined families of Dothideomycetes: Dothideomycetidae and Pleosporomycetidae

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Microfungi associated with *Clematis* (Ranunculaceae) with an integrated approach to delimiting species boundaries

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Abstract

The cosmopolitan plant genus *Clematis* contains many climbing species that can be found worldwide. The genus occurs in the wild and is grown commercially for horticulture. Microfungi on *Clematis* were collected from Belgium, China, Italy, Thailand and the UK. They are characterized by morphology and analyses of gene sequence data using an integrated species concept to validate identifications. The study revealed two new families, 12 new genera, 50 new species, 26 new host records with one dimorphic character report, and ten species are transferred to other genera. The new families revealed by multigene phylogeny are Longiostiolaceae and Pseudomassarinaceae in Pleosporales (Dothideomycetes). New genera are *Anthodidymella* (Didymellaceae), *Anthosulcatispora* and *Parasulcatispora* (Sulcatisporaceae), *Fusiformispora* (Amniculicolaceae), *Longispora* (Phaeosphaeriaceae), *Neobyssosphaeria* (Melanommataceae), *Neoleptosporella* (*Chaetosphaeriales*, genera *incertae sedis*), *Neostictis* (Stictidaceae), *Pseudohelminthosporium* (Neomassarinaceae), *Pseudomassarina* (Pseudomassarinaceae), *Sclerenchymomyces* (Leptosphaeriaceae) and *Xenoplectosphaerella* (Plectosphaerellaceae). The newly described species are *Alloleptosphaeria clematidis*, *Anthodidymella ranunculacearum*, *Anthosulcatispora subglobosa*, *Aquadictyospora clematidis*, *Brunneofusispora clematidis*, *Chaetosphaeronema clematidicola*, *C. clematidis*, *Chromolaenicola clematidis*, *Diaporthe clematidina*, *Dictyocheirospora clematidis*, *Distoseptispora clematidis*, *Floricola clematidis*, *Fusiformispora clematidis*, *Hermatomyces clematidis*, *Leptospora clematidis*, *Longispora clematidis*, *Massariosphaeria clematidis*, *Melomastia clematidis*, *M. fulvicomae*, *Neobyssosphaeria clematidis*, *Neoleptosporella clematidis*, *Neoroussoella clematidis*, *N. fulvicomae*, *Neostictis nigricans*, *Neovaginatispora clematidis*, *Parasulcatispora clematidis*, *Parathyridaria clematidis*, *P. serratifoliae*, *P. virginianae*, *Periconia verrucose*, *Phomatospora uniseriata*, *Pleopunctum clematidis*, *Pseudocapulatispora clematidis*, *Pseudocoleophoma clematidis*, *Pseudohelminthosporium clematidis*, *Pseudolophiostoma chiangraiense*, *P. clematidis*, *Pseudomassarina clematidis*, *Ramusculicola clematidis*, *Sarocladium clematidis*, *Sclerenchymomyces clematidis*, *Sigarispora clematidicola*, *S. clematidis*, *S. montanae*, *Sordaria clematidis*, *Stemphylium clematidis*, *Wojnowiciella clematidis*, *Xenodidymella clematidis*, *Xenomassariosphaeria clematidis* and *Xenoplectosphaerella clematidis*. The following fungi are recorded on *Clematis* species for the first time: *Angustimassarina rosarum*, *Dendryphion europaeum*, *Dermatiopleospora mariae*, *Diaporthe ravennica*, *D. rudis*, *Dichotomopilus ramosissimum*, *Dictyocheirospora xishuangbannaensis*, *Didymosphaeria rubi-ulmifolii*, *Fitzroyomyces cyperacearum*, *Fusarium celtidicola*, *Leptospora thailandica*, *Memnoniella oblongispora*, *Neodidymelliopsis longicolla*, *Neoeutypella baoshanensis*, *Neoroussoella heveae*, *Nigrograna chromolaenae*, *N. obliqua*, *Pestalotiopsis verruculosa*, *Pseudoberkleasmium chiangmaiense*, *Pseudoophiobolus rosae*, *Pseudoroussoella chromolaenae*, *P. elaeicola*, *Ramusculicola thailandica*, *Stemphylium vesicarium* and *Torula chromolaenae*. The new combinations are *Anthodidymella clematidis* (≡*Didymella clematidis*), *A. vitalbina* (≡*Didymella vitalbina*), *Anthosulcatispora brunnea* (≡*Neobambusicola brunnea*), *Fuscohypha kunmingensis* (≡*Plectosphaerella kunmingensis*), *Magnibotryascoma rubriostiolata* (≡*Teichospora rubriostiolata*), *Pararoussoella mangrovei* (≡*Roussoella mangrovei*), *Pseudoneoconiothyrium euonymi* (≡*Roussoella euonymi*), *Sclerenchymomyces jonesii* (≡*Neoleptosphaeria jonesii*), *Stemphylium rosae* (≡*Pleospora rosae*), and *S. rosae-caninae* (≡*Pleospora rosae-caninae*). The microfungi on *Clematis* is distributed in several classes of

Extended author information available on the last page of the article

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The numbers of fungi: is the descriptive curve flattening?

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Abstract
The recent realistic estimate of fungal numbers which used various algorithms was between 2.2 and 3.8 million. There are nearly 100,000 accepted species of Fungi and fungus-like taxa, which is between 2.6 and 4.5% of the estimated species. Several forums such as Botanica Marina series, Fungal Diversity notes, Fungal Biodiversity Profiles, Fungal Systematics and Evolution—New and Interesting Fungi, Mycosphere notes and Fungal Planet have enhanced the introduction of new taxa and nearly 2000 species have been introduced in these publications in the last decade. The need to define a fungal species more accurately has been recognized, but there is much research needed before this can be better clarified. We address the evidence that is needed to estimate the numbers of fungi and address the various advances that have been made towards its understanding. Some genera are barely known, whereas some plant pathogens comprise numerous species complexes and numbers are steadily increasing. In this paper, we examine ten genera as case studies to establish trends in fungal description and introduce new species in each genus. The genera are the ascomycetes *Colletotrichum* and *Pestalotiopsis* (with many species or complexes), *Atrocalyx*, *Dothiora*, *Lignosphaeria*, *Okeanomyces*, *Rhamphoriopsis*, *Thozetella*, *Thyrostroma* (relatively poorly studied genera) and the basidiomycete genus *Lepiota*. We provide examples where knowledge is incomplete or lacking and suggest areas needing further research. These include (1) the need to establish what is a species, (2) the need to establish how host-specific fungi are, not in highly disturbed urban areas, but in pristine or relatively undisturbed forests, and (3) the need to establish if species in different continents, islands, countries or regions are different, or if the same fungi occur worldwide? Finally, we conclude whether we are anywhere near to flattening the curve in new species description.

Keywords 11 new taxa · *Atrocalyx* · *Colletotrichum* · *Dothiora* · fungal numbers · Host-specificity · *Lepiota* · *Lignosphaeria* · *Okeanomyces* · *Pestalotiopsis* · *Rhamphoriopsis* · *Thozetella* · *Thyrostroma*

Introduction

The estimated numbers of fungi have always been a compelling topic with numerous discussion papers over time. Mycologists have always pondered over how many fungal species there are, as this has important implications for conservation practices. Fungi, including the seen mushrooms and unseen microorganisms are diverse and form an integral component of life's genetic diversity, but their actual numbers are poorly understood and the estimates available so far are debatable. Hawksworth (1991) provide a comprehensive account and argument for the numbers being 1.5 million. This was based on, amongst other metrics, there being circa six taxa as unique to each plant species. Since this critical paper, there have been several revisions (Table 1) with estimates ranging from 0.5 to 13.2 million, and the latest estimate being 11.7–13.2 million (Wu et al. 2019). Hawksworth and Lücking (2017) based their estimates (2.2–3.8 M), taking into account cryptic species, the rates and patterns at which new species are being described, unexplored niches and DNA based species from environmental DNA. New generation sequencing has also provided

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Pseudocercospora dypsidis sp. nov. (Mycosphaerellaceae) on *Dypsis lutescens* leaves in Thailand

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Abstract

A cercosporoid fungus associated with leaf lesions of Dypsis lutenscens (Arecaceae) was observed in Chiang Rai, Thailand. This study describes the new species Pseudocercospora dypsidis based on morphological characteristics and phylogenetic analyses from three gene regions, ITS, TEF-1α, and ACT. Single gene analyses are generally insufficient for identification of Pseudocercospora species, or for segregation of other genera in the Pseudocercospora complex. A list is provided of all Pseudocercospora species known from Thailand and the need to confirm their identifications by a polyphasic approach is stressed.

Key words: new species, multigene analyses, Mycosphaerella, palm, Pseudocercospora spp. in Thailand

Introduction

Palms are one of the most important ornamental tree groups with approximately 3,000 species (Benítez & Soto 2010). Areca palm (*Dypsis lutescens*), a member of Arecaceae, has a worldwide distribution and is used for landscaping and decoration. *Dypsis lutescens* prefers warm and humid sub-tropical to tropical climates, conditions that are favorable for many diseases (Basu & Mondol 2012). As ornamental palms have a high aesthetic value, foliar diseases may cause economic loss (Elliott *et al.* 2004). In July 2019, numerous plants were observed with widespread leaf lesions in Mae Fah Luang University, Chiang Rai Province, Thailand; these lesions were shown to be caused by a *Pseudocercospora* sp.

Pseudocercospora typified by *P. vitis* was introduced by Spegazzini (1910). *Pseudocercospora* are asexual fungi which belong to Mycosphaerecellaceae (Capnodiales, Dothideomycetes), closely related to mycosphaerella-like sexual morphs (Braun *et al.* 2013, 2014, 2015, Crous *et al.* 2013a, Silva *et al.* 2016). Species of *Pseudocercospora* are identified mainly from tropical and sub-tropical regions (Wanasinghe *et al.* 2018). They cause leaf spots, blight, and necrotic lesions on flowers and fruits of various cultivated and native plants (Chupp 1954, Agrios 2005, Crous & Braun 2003). The typical characteristic of leaf spots caused by these species are distinct chlorotic margins that demarcate the lesions (Crous *et al.* 2013a). These fungi typically have scolecosporous conidia and dematiaceous conidiophores with unthickened and not darkened conidiogenous loci and hila (Crous & Braun 2003, Braun *et al.* 2013). The present study aims to identify the causal agent associated with the foliar infection on *Dypsis lutescens* observed in Chiang Rai, Thailand using morphological and molecular methods.

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One stop shop IV: taxonomic update with molecular phylogeny for important phytopathogenic genera: 76–100 (2020)

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Abstract
This is a continuation of a series focused on providing a stable platform for the taxonomy of phytopathogenic fungi and fungus-like organisms. This paper focuses on one family: *Erysiphaceae* and 24 phytopathogenic genera: *Armillaria, Barriopsis*, *Cercospora*, *Cladosporium*, *Clinoconidium*, *Colletotrichum*, *Cylindrocladiella*, *Dothidotthia*,, *Fomitopsis*, *Ganoderma*, *Golovinomyces*, *Heterobasidium*, *Meliola*, *Mucor*, *Neoerysiphe*, *Nothophoma*, *Phellinus*, *Phytophthora*, *Pseudoseptoria*, *Pythium*, *Rhizopus*, *Stemphylium*, *Thyrostroma* and *Wojnowiciella*. Each genus is provided with a taxonomic background, distribution, hosts, disease symptoms, and updated backbone trees. Species confirmed with pathogenicity studies are denoted when data are available. Six of the genera are updated from previous entries as many new species have been described.

Keywords Disease · Plant pathology · Phylogeny · Taxonomy · Symptoms

Contents and contributors (main contributors underlined)

Newly discussed genera and family

76. *Armillaria* – B Chuankid, M Stadler
77. *Barriopsis* – IS Manawasinghe, RS Jayawardena
78. *Cercospora* – ID Goonasekara
79. *Clinoconidium* – AK Gautam, S Avasthi
80. *Cylindrocladiella* – D Harischandra, RS Jayawardena
81. *Dothidotthia* – C Senwanna
82. *Erysiphaceae* – KK Liyanage, RS Jayawardena, KD Hyde
83. *Fomitopsis* – V Papp, B Palla, D Papp
84. *Ganoderma* – KK Hapuarachchi, T Luangharn, O Raspe
85. *Golovinomyces* – RS Jayawardena
86. *Heterobasidium* – V Papp, B Palla, D Papp
87. *Meliola* – S Hongsanan, XY Zeng
88. *Neoerysiphe* – RS Jayawardena
89. *Nothophoma* – IS Manawasinghe, RS Jayawardena
90. *Phellinus* – V Papp, B Palla, D Papp
91. *Pseudoseptoria* – A Karunarathna, RS Jayawardena
92. *Stemphylium* – RS Jayawardena, KD Hyde
93. *Thyrostroma* – C Senwanna, KD Hyde
94. *Wojnowiciella* – D Harischandra, RS Jayawardena

Updated genera

95. *Cladosporium* – NG Liu, RS Jayawardena
96. *Colletotrichum* – RS Jayawardena, KD Hyde
97. *Mucor* – VG Hurdeal, HB Lee
98. *Phytophthora* – CS Bhunjun, RS Jayawardena
99. *Pythium* – CS Bhunjun, RS Jayawardena
100. *Rhizopus* – VG Hurdeal, HB Lee

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Article
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Applied aspects of methods to infer phylogenetic relationships amongst fungi

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Abstract

There is a need to document the methodologies used for molecular phylogenetic analyses since the current fungal identification, classification and phylogeny are necessarily applied with DNA molecular sequence data. Hence this manuscript is mainly aimed to provide a basic reference or guideline for the mycologists venturing into the field of phylogenetic studies and to avoid unnecessary repetitions in related publications. This is not at all to elaborate the theoretical background but is intended as a compact guide of some useful software references and coding for the most commonly used phylogenetic methods applied in mycological research. This reference manuscript is originally conceived for usage with a set of programs necessary for inference of phylogenetic analyses in fungal research (taxonomy and phylogeny). For this purpose, principles, introductory texts and formulas have been omitted. Meanwhile, necessary steps from DNA extraction to DNA sequencing, sequences quality check, data alignment and general steps of phylogenetic tree reconstructions have been included.

Keywords – Fungal classification – Materials and methodology – Molecular data – Reference – Taxonomy

Introduction

A 'classification system' refers to placing organisms within hierarchical groups (e.g. species, genus) and aggregating them to form supergroups of upper rank (e.g. family, order) forming a taxonomic grading (Judd et al. 2007, Simpson & Michael 2010, Liu et al. 2017). Based on shared characteristics, 'taxonomy' defines clusters of biological organisms by naming those groups (Judd et al. 2007, Simpson & Michael 2010). Taxonomy is usually based on phylogenetic relationships, placing similar taxa together by organizing them into monophyletic groups. Formerly, organisms were classified based on morphological comparisons as advanced molecular techniques were not available to support traditional species classification (Guarro et al. 1999). This artificial classification scheme led to taxonomic disagreements.

'Mycology' is a comparatively young scientific field which developed with the innovation of the microscope in the 17th century. The inspiring work of the mycological history can be exemplified by the findings in Pier Antonio Micheli's book 'Nova plantarum genera' in 1729 (Alexopoulos 1963) which were based on the observation of fungal spores. Hendrik Persoon

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Morphological approaches in studying fungi: collection, examination, isolation, sporulation and preservation

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Abstract

Traditionally, fungal taxonomy was based on observable phenotypic characters. Recent advances have driven taxonomic conclusions towards DNA-based approaches and these techniques have corresponding pros and cons. Species concepts must therefore rely on incorporated approaches of genotypic, phenotypic and physiological characters and chemotaxonomy. Examination and interpretation of morphological characters however vary from person to person. Standardized procedures are used in the taxonomic study of fungi and general practices of phenotypic approaches are herein outlined. It is not possible to detail all techniques for all fungi and thus, this paper emphasizes on microfungi. Specimen collection is the initial step in any

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Refined families of Dothideomycetes: orders and families incertae sedis in Dothideomycetes

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Abstract

Numerous new taxa and classifications of Dothideomycetes have been published following the last monograph of families of Dothideomycetes in 2013. A recent publication by Honsanan et al. in 2020 expanded information of families in Dothideomycetidae and Pleosporomycetidae with modern classifications. In this paper, we provide a refined updated document on orders and families *incertae sedis* of Dothideomycetes. Each family is provided with an updated description, notes, including figures to represent the morphology, a list of accepted genera, and economic and ecological significances. We also provide phylogenetic trees for each order. In this study, 31 orders which consist 50 families are assigned as orders *incertae sedis* in Dothideomycetes, and 41 families are treated as families *incertae sedis* due to lack of molecular or morphological evidence. The new order, Catinellales, and four new families, *Catinellaceae*, *Morenoinaceae Neobuelliellaceae* and *Thyrinulaceae* are introduced. Seven genera (*Neobuelliella*, *Pseudomicrothyrium*, *Flagellostrigula*, *Swinscowia*, *Macroconstrictolumina*, *Pseudobogoriella*, and *Schummia*) are introduced. Seven new species (*Acrospermum urticae*, *Bogoriella complexoluminata*, *Dothiorella ostryae*, *Dyfrolomyces distoseptatus*, *Macroconstrictolumina megalateralis*, *Patellaria microspora*, and *Pseudomicrothyrium thailandicum*) are introduced base on morphology and phylogeny, together with two new records/reports and five new collections from different families. Ninety new combinations are also provided in this paper.

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Extended author information available on the last page of the article

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Integrating Different Lines of Evidence to Establish a Novel Ascomycete Genus and Family (*Anastomitrabeculia*, *Anastomitrabeculiaceae*) in *Pleosporales*

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Abstract: A novel genus, *Anastomitrabeculia*, is introduced herein for a distinct species, *Anastomitrabeculia didymospora*, collected as a saprobe on dead bamboo culms from a freshwater stream in Thailand. *Anastomitrabeculia* is distinct in its trabeculate pseudoparaphyses and ascospores with longitudinally striate wall ornamentation. A new family, *Anastomitrabeculiaceae*, is introduced to accommodate *Anastomitrabeculia*. *Anastomitrabeculiaceae* forms an independent lineage basal to *Halojulellaceae* in *Pleosporales* and it is closely related to *Neohendersoniaceae* based on phylogenetic analyses of a combined *LSU*, *SSU* and *TEF1α* dataset. In addition, divergence time estimates provide further support for the establishment of *Anastomitrabeculiaceae*. The family diverged around 84 million years ago (MYA) during the Cretaceous period, which supports the establishment of the new family. The crown and stem age of *Anastomitrabeculiaceae* was also compared to morphologically similar pleosporalean families.

Keywords: BEAST; *Dothideomycetes*; *Pleosporales*; *Poaceae*; taxonomy; three new taxa; trabeculate pseudoparaphyses

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1. Introduction

Pleosporales is the largest order within *Dothideomycetes* (*Ascomycota*) [1]. The taxonomic and phylogenetic relationships of families and genera within this order are well documented [1–7]. *Pleosporales* comprises two suborders, *Massarineae* and *Pleosporineae* [1]. *Pleosporineae* includes economically important plant pathogens and *Massarineae* includes mainly saprobes from terrestrial or aquatic environments [1,3]. Zhang et al. [1] revised 174 genera and accepted 26 families in *Pleosporales*. The suborder *Massarineae* was resurrected to accommodate five families, the *Lentitheciaceae*, *Massarinaceae*, *Montagnulaceae* (*Didymosphaeriaceae*), *Morosphaeriaceae* and *Trematosphaeriaceae* [1]. Hyde et al. [2] correlated morphology with phylogenetic evidence and accepted 41 families in this order. Tanaka et al. [3] introduced two new families, *Parabambusicolaceae* and *Sulcatisporaceae*, accepting 12 families in *Massarineae*. The family *Longipedicellataceae* was introduced, and the divergence time in *Pleosporales* was estimated with emphasis on *Massarineae* [4]. The crown age of *Pleosporales* was dated to 211 MYA and *Massarineae* was dated to 130 MYA [4]. Species boundaries in *Cucurbitariaceae* were revised [5] and the family, *Lentimurisporaceae*, was introduced in *Pleosporales* [6].

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Investigating species boundaries in *Colletotrichum*

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Abstract

Colletotrichum is one of the most important plant pathogenic genera that is responsible for numerous diseases which can have a profound impact on the agricultural sector. Species delineation is difficult due to a lack of distinctive phenotypic variation. Therefore, in this study three different genomic approaches based on phylogenetic, evolutionary and coalescent-based methods are applied to establish robust species boundaries. The reliability of five different DNA barcodes was also assessed to provide further insights into species delineation. The ITS region can resolve the placement of taxa up to the species complex level. The *GAPDH* and *TUB2* markers are determined to be the most informative for most complexes. However, no single marker could discriminate between species in all complexes, therefore different molecular approaches based on multi-locus datasets are recommended. This is the first study to provide an estimated divergence time for all species complexes in *Colletotrichum*. The estimated divergence time for species complexes ranged between 4.8 to 32.2 MYA. Based on the high level of congruent results obtained from the different molecular approaches, a new species complex, the *Colletotrichum agaves* complex is introduced. This complex consists of five taxa which are characterised by the presence of straight or slightly curved conidia with obtuse apices. This study shows that coalescent approaches and multi-locus phylogeny are crucial to establish species boundaries in *Colletotrichum*. The taxonomic placement of three singleton taxa *Colletotrichum axonopodi*, *C. cariniferi* and *C. parallelophorum* is revised. We accept 248 species and provide recommendations regarding species boundaries in the graminicola–caudatum complex.

Keywords BEAST · General mixed yule coalescent method · *Glomerellaceae* · Multi-rate poisson tree process · *Sordariomycetes* · Taxonomy

Introduction

Colletotrichum was introduced by Corda (1831) with *C. lineola* as the type species and it is the only member of *Glomerellaceae* (*Glomerellales*, *Sordariomycetes*) (Maharachchikumbura et al. 2016; Hyde et al. 2019, 2020b). *Colletotrichum* species are endophytes, pathogens and saprobes (Tao et al. 2013; Hyde et al. 2014; Jayawardena et al. 2016a, b; Rashmi et al. 2019). As endophytes, they are one of the most widely distributed genera and can produce a range of secondary metabolites (Moraga et al. 2019). As plant pathogens, species can cause anthracnose and postharvest fruit rots (Phoulivong et al. 2010), leading to yield loss especially in high-value crops worldwide. Some species are associated with a single host while others can infect multiple hosts, and this hinders effective disease management (da Silva et al. 2020). *Colletotrichum* species have also been known to cause human infections for example, *C. dematium*,

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Structure and Development of Ascomata

Chitrabhanu S. Bhunjun, Chayanard Phukhamsakda, Kevin D. Hyde

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Abstract

Sexual reproduction in fungi results in the production of highly complex structures that contain specialized sexual tissues called ascomata. Ascomata formation is a complex process which requires the expression of specific genes and metabolic pathways that can be affected by environmental factors. Filamentous ascomycetes serve as an important model to study various important processes including the development of ascomata. In this article, we provide an overview of the morphology of ascomata and its development. We also discuss factors that can control the development of ascomata and provide an overview of the some of the genes involved in the process.

Keywords

Development; Environmental stresses; Filamentous ascomycetes; Morphology; Regulatory networks; Sexual reproduction

Fungal Diversity
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REVIEW

What are fungal species and how to delineate them?

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Abstract
This is the opening paper in the special issue of *Fungal Diversity*, which collates the data on defining species. Defining and recognizing species has long been a controversial issue. Since Darwin's proposed origin of species, over 30 species criteria have been brought forth and used to define species boundaries. In recent times, phylogenetic analyses based on multiple loci have been extensively used as a method to define species boundaries. However, only a few mycologists are aware that phylogenetic species criteria can mask discordances among fungal groups, leading to inaccurately defined species boundaries. In the current review, we discuss species recognition criteria, how and where these criteria can be applied along with their limitations and derived alternatives. In order to delimit fungal species, authors need to take into account not only the phylogenetic and phenotypic coherence, but also the timing of events that lead to fungal speciation and subsequent diversifications. Variations in the rate of phenotypic diversifications and convergent fungal evolution make it difficult to establish a universal species recognition criterion. The best practice can only be defined in the context of each fungal group. In this review, we provide a set of guidelines, encouraging an integrative taxonomic approach for species delimitation that can be used to define fungal species boundaries in the future. The other papers in this special issue deal with fungal speciation in *Ascomycota*, *Dothideomycetes*, *Basidiomycota*, basal fungi, lichen-forming fungi, plant pathogenic fungi, and yeasts.

Keywords Allopatry · Speciation · Species concepts · Species criteria · Species recognition · Sympatry

Introduction

This paper is the introductory paper of this special issue entitled "What is a Species?", which is aimed at discussing mechanisms of fungal speciation, and conceptual frameworks and associated metrics for fungal delimitation. The current review discusses fungal speciation, the applicability of existing species criteria in defining species boundaries and possible adaptations to the fungal nomenclature with omics data. Considering different species recognition criteria, we recommend a set of approaches for defining species under the existing criteria. In other contributions to this special issue, Maharachchikumbura and co-authors review the current species recognition criteria used for *Ascomycota* with their advantages and disadvantages. Furthermore, they outline the drawbacks in the traditional phylogenetic methods that lead to ambiguous conclusions and propose an integrative and pragmatic approach for identifying species boundaries in *Ascomycota*. The ecology, taxonomy and diversity of the largest and most diverse class in *Ascomycota*, *Dothideomycetes*, is discussed by Pem and co-authors. They discuss the current species criteria used to define species boundaries in several highly confused fungal taxa and provide basic guidelines for standard *Dothideomycetes* taxonomy based on morphology and phylogeny. Cao and co-authors discuss the species recognition criteria used in *Basidiomycota* together with the difficulties faced during species recognition and present recommendations for species delimitations. Species recognition criteria applicable

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Article
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Colletotrichum: lifestyles, biology, morpho-species, species complexes and accepted species

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Abstract

Colletotrichum is an important plant pathogenic genus that has undergone tremendous taxonomic changes. Species of *Colletotrichum* also occur as endophytes, saprobes and rarely entomopathogens. The current understanding of *Colletotrichum* taxonomy and application of various techniques in defining species within the genus is discussed in this paper. Here we provide a review of lifestyles, infection mechanisms, life cycle, host-specificity, classification history and techniques defining *Colletotrichum* species and the relation to speciation. Misidentifications and mistakes during species introduction are discussed and recommendations are provided for valid species publication. We provide an account of 248 currently accepted species with molecular data, which falls into 14 species-complexes and 13 singleton species. An updated account of *Colletotrichum* species is provided. Species are listed alphabetically in each species complex and annotated with their habitat, host, geographic distribution, phylogenetic position and typification details. Tables of host specific (152 species) and geographically endemic *Colletotrichum* species (19 species) are provided. A table of 450 morpho-species is provided for the first time gathering all data to one place, allowing mycologists to check before publishing a new species. Phylogenetic trees are provided for the whole genus and each species complex. Genes and combinations of genes that can be used for the identification of the species complexes are suggested. Future directions for the advancement of this genus are discussed.

Keywords – Host-specificity – Morpho-species – Pathogens – Singleton – Taxonomy

Introduction

Colletotrichum is the sole member of *Glomerellaceae* (Glomerellales, Sordariomycetes), and was introduced by Corda (1831) (Réblová et al. 2011, Maharachchikumbura et al. 2015, 2016, Hyde et al. 2020). Many species of this genus are important pathogens, while some are endophytes and saprobes (Cannon et al. 2012, Hyde et al. 2014, 2020, Jayawardena et al. 2016a, 2020). At the time of the first monographic treatment of *Colletotrichum* (von Arx 1957b), around 750 names existed (Cannon et al. 2012). von Arx (1957b) reduced this to 11 taxa based on morphological characters. Sutton (1980) accepted 22 species, while Sutton (1992) accepted 39 species based on

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REVIEW

What is a species in fungal plant pathogens?

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Abstract
Scientific names are crucial for communicating knowledge concerning fungi and fungus-like organisms. In plant pathology, they link information regarding biology, host range, distribution and potential risk to agriculture and food security. In the past, delimitation among pathogenic taxa was primarily based on morphological characteristics. Due to distinct species sharing overlapping characteristics, the morphological identification of species is often neither straightforward nor reliable. Hence, the phylogenetic species concept based on molecular phylogenetic reconstructions gained importance. The present opinion discusses what a fungal species is and how identification of species in plant pathology has changed over the past decades. In this context, host-specialization and species complexes are discussed. Furthermore, species concepts in plant pathology are examined using case studies from *Bipolaris*, *Colletotrichum*, *Curvularia*, *Diaporthe*, *Diplodia*, *Meliola*, *Plasmopara*, rust fungi and *Trichoderma*. Each entry contains a brief introduction to the genus, concepts used in species identification so far and the problems in describing a species followed by recommendations. The importance of correctly naming and identifying a species is addressed in the context of recent introductions, and we also discuss whether the introduction of new species in pathogenic genera has been overestimated. We also provide guidelines to be considered when introducing a new species in a plant pathogenic genus.

Keywords Dual nomenclature · Host-specificity · Polyphasic approach · Pathology · Phylogeny · Species delimitation · Systematics

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pathogens

MDPI

Review

Importance of Molecular Data to Identify Fungal Plant Pathogens and Guidelines for Pathogenicity Testing Based on Koch's Postulates

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Abstract: Fungi are an essential component of any ecosystem, but they can also cause mild and severe plant diseases. Plant diseases are caused by a wide array of fungal groups that affect a diverse range of hosts with different tissue specificities. Fungi were previously named based only on morphology and, in many cases, host association, which has led to superfluous species names and synonyms. Morphology-based identification represents an important method for genus level identification and molecular data are important to accurately identify species. Accurate identification of fungal pathogens is vital as the scientific name links the knowledge concerning a species including the biology, host range, distribution, and potential risk of the pathogen, which are vital for effective control measures. Thus, in the modern era, a polyphasic approach is recommended when identifying fungal pathogens. It is also important to determine if the organism is capable of causing host damage, which usually relies on the application of Koch's postulates for fungal plant pathogens. The importance and the challenges of applying Koch's postulates are discussed. Bradford Hill criteria, which are generally used in establishing the cause of human disease, are briefly introduced. We provide guidelines for pathogenicity testing based on the implementation of modified Koch's postulates incorporating biological gradient, consistency, and plausibility criteria from Bradford Hill. We provide a set of protocols for fungal pathogenicity testing along with a severity score guide, which takes into consideration the depth of lesions. The application of a standard protocol for fungal pathogenicity testing and disease assessment in plants will enable inter-studies comparison, thus improving accuracy. When introducing novel plant pathogenic fungal species without proving the taxon is the causal agent using Koch's postulates, we advise the use of the term *associated with the "disease symptoms" of "the host plant"*. Where possible, details of disease symptoms should be clearly articulated.

Keywords: disease severity; image analysis; pathogenicity; phylogeny; plant disease assessment

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1. Introduction

Animals and plants live in close contact with innumerable microorganisms, but only a small percentage can cause disease [1]. Fungi exhibit different types of associations with plants ranging from mutualism to parasitism [2]. They are vital in nutrient cycling which releases key plant nutrients into the soil [3]. As plant pathogens, they can cause significant damage in agriculture and forestry [4]. It was assumed that pathogenic microorganisms

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