A polyphasic approach for studying *Colletotrichum*

Cai, L.[1*], Hyde, K.D.[2,3], Taylor, P.W.J.[4], Weir, B.S.[5], Waller, J.M.[6], Abang, M.M.[7], Zhang, J.Z.[8], Yang, Y.L.[9], Phoulivong, S.[3], Liu, Z.Y.[9], Prihastuti, H.[3], Shivas, R.G.[10], McKenzie, E.H.C.[5] and Johnston, P.R.[5]

[1]Novozymes China, No. 14, Xinxi Road, Shangdi, HaiDian, Beijing, 100085, PR China
[2]International Fungal Research & Development Centre, The Research Institute of Resource Insects, Chinese Academy of Forestry, Bailongsi, Kunming 650224, PR China
[3]School of Science, Mae Fah Luang University, Thasud, Chiang Rai 57100, Thailand
[4]BioMarka/Center for Plant Health, Melbourne School of Land and Environment, The University of Melbourne, Victoria 3010 Australia
[5]Landcare Research New Zealand Ltd, Private Bag 92170, Auckland Mail Centre, Auckland 1142, New Zealand
[6]CABI Europe UK, Bakeham Lane, Egham, Surrey, TW20 9TY.
[7]AVRDC-The World Vegetable Center, Regional Center for Africa, PO Box 10 Duluti, Arusha, Tanzania
[8]Institute of Biotechnology, College of Agriculture & biotechnology, Zhejiang University, Kaixuan Rd 258, Hangzhou 310029, PR China
[9] Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou 550006 P.R. China
[10]Plant Pathology Herbarium, Queensland Primary Industries and Fisheries, 80 Meiers Road, Indooroopilly 4068, Queensland, Australia

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Colletotrichum is the causal agent of anthracnose and other diseases on leaves, stems and fruits of numerous plant species, including several important crops. Accurate species identification is critical to understand the epidemiology and to develop effective control of these diseases. Morphologically-based identification of *Colletotrichum* species has always been problematic, because there are few reliable characters and many of these characters are plastic, dependent upon methods and experimental conditions. Rapid progress in molecular phylogenetic methods is now making it possible to recognise stable and well-resolved clades within *Colletotrichum*. How these should be reflected in a classification system remains to be resolved. An important step in providing a stable taxonomy for the genus is to epitypify existing names, and in so doing link them to genetically defined clades. We recommend a polyphasic approach to the recognition and identification of species within *Colletotrichum,* matching genetic distinctness with informative morphological and biological characters. This paper reviews various approaches in the study of *Colletotrichum* complexes including morphology, pathogenicity, physiology, phylogenetics and secondary metabolite production. A backbone phylogenetic tree using ITS sequence data from 42 ex-type specimens has been generated. Phylogenetic analysis using ITS sequence data is a useful tool to give a preliminarily identification for *Colletotrichum* species or place them in species complexes. However, caution must be taken here as the majority of the ITS sequences deposited in GenBank are wrongly named. Multi-gene phylogenetic data provides much better understanding of the relationships within *Colletotrichum* and should be employed where possible. We propose that an ideal approach for *Colletotrichum* systematics should be based on a multi-gene phylogeny, with comparison made with type specimens, and a well-defined phylogenetic lineage should be in conjunction with recognisable polyphasic characters, such as morphology, physiology, pathogenicity, cultural characteristics and secondary metabolites. Finally a set of protocols and methodologies is provided as a guideline for future studies, epitypification and the description of new species.

Key words: barcoding, epitypification, molecular phylogeny, morphology, pathogenicity, physiology, systematics

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*****Corresponding author: mrcailei@gmail.com

Introduction

The need for species recognition

Colletotrichum Corda is one of the economically most important genera of fungi, being responsible for anthracnose and other diseases of a wide range of plant species (Sutton, 1980; Hyde *et al*., 2009). The importance of a rigid and stable taxonomy for the determination of *Colletotrichum* species is, therefore, a significant practical concern (Shenoy *et al*., 2007). However, there has been considerable difficulty surrounding *Colletotrichum* systematics, due to the lack of reliable morphological features, making species boundaries ambiguous and confusing. Traditionally many *Colletotrichum* species have been named after their host, which suggests host specificity amongst species. In what some considered a drastic move, von Arx (1957) reduced the number of *Colletotrichum* species from several hundred to 11 based on morphological characters, with many taxa treated as synonyms of *C. gloeosporioides* and *C. dematium*. Several additional species have been since accepted, based on morphological criteria (Sutton, 1980; 1992).

The difficulty in recognising *Colletotrichum* species has resulted from: 1) few and variable morphological characters; 2) an extensive host range and variability in pathogenicity (Bailey and Jeger, 1992; Freeman *et al*., 2000; Latunde-Dada, 2001; Du *et al*., 2005; Thaung, 2008); 3) type specimens are often missing or in poor condition thus they can not be used for molecular study and; 4) numerous rDNA ITS (ITS) and other sequences of *Colletotrichum* strains in NCBI are often erroneously named (Crouch *et al*., 2009d; Damm *et al*., 2009). To solve the problem of few morphologically informative characters, researchers have utilised other characters such as nucleic acid sequence data, physiology, secondary metabolites and pathogenicity, as part of a polyphasic approach (Sutton, 1992; Cannon *et al*. 2000; Than *et al*., 2008a,b; Crouch *et al*., 2009b; Prihastuti *et al*., 2009). Phylogenetic analysis based on nucleic acid sequences have been successfully used to differentiate species in other difficult genera or groups (e.g. *Fusarium*—Shenoy *et al*., 2007; Kvas *et al*., 2009; O'Donnell *et al*., 2009; *Botryosphaeriaceae*—Slippers *et al*., 2004a,b; Crous *et al*., 2006; Alves *et al*., 2006, 2008; Phillips *et al*., 2007); although unsuccessfully in other genera (e.g. *Pestalotiopsis*—Jeewon *et al*., 2002, 2003; Lee *et al*., 2006; Hu *et al*., 2007; Tesjevi *et al*., 2007). Such data is especially important in species differentiation and understanding the species relationships in *Colletotrichum*.

One potential solution for poor type material is to designate an epitype for the species; this stabilises the species name and should provide sequence data as well as living specimens for future research (Hyde and Zhang, 2008). Thirteen species of *Colletotrichum* have been epitypified and/or newly combined in the genus (Crouch *et al*., 2006; Shenoy *et al*., 2007; Cannon *et al*., 2008; Than *et al*., 2008a; Crouch *et al*., 2009b; Damm *et al*., 2009; Weir and Johnston, in press), while recently described new species have ex-type cultures (Crouch *et al*., 2009b; Damm *et al*., 2009; Prihastuti *et al*., 2009; Shivas and Tan, 2009; Yang *et al*., 2009). The use of sequences from type specimens is essential when diagnosing *Colletotrichum* species or studying their phylogenetic relationships. Where possible, all previously designated *Colletotrichum gloeosporioides* strains should be compared against the recently designated epitype of *C. gloeosporioides* (Cannon *et al*., 2008) to establish if they are correctly named.

Colletotrichum taxonomy is presently unsatisfactory and there is a pressing need for a polyphasic approach for identification, which reflects the natural classification of species and subspecific taxa within the genus (Sutton, 1992; Cannon *et al*., 2000). In this paper a taxonomic framework for describing *Colletotrichum* epitypes and new species is proposed. This utilises a multi-gene phylogeny based on type specimens, and well-defined phylogenetic lineages which correlate with recognisable polyphasic characters such as morphology, cultural characteristics, physiology and pathogenicity. The various approaches that have been used or could be incorporated in the study of *Colletotrichum* species, with recommended methodologies and discussion on correlation of characters with phylogenetic types are discussed. A phylogenetic analysis based on the internal transcribed spacers and the 5.8S ribosomal RNA gene (ITS) from specimens including 42 ex-type strains is also provided (Fig. 1).

Established methodology

Morphology

Traditional identification systems in *Colletotrichum* have relied heavily on morphological and cultural characteristics (von Arx, 1957; Sutton, 1980, 1992; Bailey and Jeger, 1992; Freeman *et al*., 1998). Sutton (1992) noted that morphology alone does not provide sufficient information for a precise identification, especially for those species in the *C. gloeosporioides* and *C. dematium* complexes. Crouch *et al*. (2009b) considered that conidial size and shape, along with conidial appressoria were taxonomically uninformative and of little use for species diagnosis in graminicolous *Colletotrichum* species. Species with similar morphological characteristics may have considerable variation at the physiological and pathogenic levels. Recognition of this is particularly important for biosecurity, plant breeding and integrated disease management. Taxonomy based on morphology alone is likely to result in ambiguity. Recent studies have shown that morphological characters should be used in conjunction with other characters to establish species relationships within *Colletotrichum* (Crouch *et al*., 2009b; Prihastuti *et al*., 2009).

Available morphological characters of *Colletotrichum* species include: 1) characters on natural substrata, i.e. size and shape of conidiomata (acervuli), conidia, conidiophores and setae; 2) size and shape of conidia, conidiophores and setae in culture; and 3) size and shape of appressoria. Acervuli, setae and conidial characters (shape and dimensions) on natural substrata can vary due to environmental factors, and conidia may be absent from infected host tissues. Some species of *Colletotrichum*, e.g. *C. musae* and *C. gossypii*, consistently fail to produce setae in conidiomata (Sutton and Waterston, 1970) and their presence on natural hosts is often inconsistent for species diagnosis (von Arx, 1957; Sutton, 1966).

Morphological characters may vary with environmental factors, and incubation conditions, such as media and temperature, should be standardized (Cannon *et al*., 2000) for species comparison and identification. Unfortunately, there is no recommended standard for *Colletotrichum* cultivation. For example, Baxter *et al*. (1983) described species of *Colletotrichum* based on growth on MSA and CDY at 20ºC; Sutton (1980) used potato dextrose agar (PDA) under alternating 12 hours near ultra violet irradiation / 12 hour dark at 25ºC; others have used PDA in the dark at 25ºC (Shivas *et al*., 1998; Nirenberg *et al*., 2002) or; synthetic nutrient-poor agar (SNA) medium and *Anthriscus* stems incubated under permanent near ultra violet light at 20 ºC (Damm *et al*., 2009). Thus, it is difficult to compare morphological and cultural characters due to variation and inconsistency in methodology. To compare isolates/species, a standardised protocol for cultivation of strains should be applied.

Observations and measurements of conidial size and shape have usually been made using conidial masses mounted in water, cotton blue, or lactic acid (Cannon *et al*., 2008). It should be noted that many species of *Colletotrichum* produce secondary conidia in culture directly from germinated primary conidia; these may vary morphologically when compared to those produced in conidiomata (Cannon *et al*., 2000).

Conidial and mycelial appressoria (also referred to as hyphopodia) are often described in *Colletotrichum* species. Sutton (1980) characterised the mycelial appressoria formed in potato-carrot agar (PCA) using a slide culture technique, while others have used PDA with incubation in the dark (Johnston and Jones, 1997; Malloch, 1981) or have observed mycelial appressoria formed on the undersurface of SNA cultures (Damm *et al*., 2009). Conidial appressoria have been recorded when conidia are germinated in drops of deionised water on plastic cover slips in a moist chamber (Johnson *et al*., 1997; Chaky *et al*., 2001). Conidial appressoria are taxonomically uninformative and of little use for species diagnosis (Sutton, 1980, 1992). However, Du *et al.* (2005) noted that the shape of appressoria can differentiate *C. acutatum* (with rather smooth appressoria) from *C. gloeosporioides* (with more lobed appressoria). Crouch *et al*. (2009b) found that appressoria shape and size are useful for delimiting grass-associated *Colletotrichum* species, but should be used in combination with host range. Yang *et al*. (2009) found that conidial appressoria can distinguish two species of *Colletotrichum* from *Amaryllidaceae*. The conidial appressoria of *C. hippeastri* are larger than those of *C. hymenocallidis* and are irregular, crenate or lobed, occasionally becoming complex. In *C. hymenocallidis* they are ovate or sometimes clavate

Cultural characteristics on agar media have been applied for the diagnosis of some *Colletotrichum* species (von Arx, 1957). The conidial morphology of *C. gloeosporioides* and *C. lindemuthianum* are similar, but their cultural characters are distinctly different. *Colletotrichum lindemuthianum* produces dark pigmentation in media and grows consistently slower than *C. gloeosporioides* (Baxter *et al*., 1983; Sutton, 1992). Likewise, *C. musae* grows relatively fast, forming effuse colonies and glabrous conidiomata with many conidia, while *C. destructivum* forms large conidiogenous zones with scattered, relatively short setae and has limited conidial production (Baxter *et al*., 1983).

The shape and size of conidia and appressoria, as well as cultural characters should be evaluated and used with caution, as these characters are highly dependent on the growth conditions. Erroneous diagnosis can largely be avoided if morphology is used in conjunction with other characters such as molecular sequence data, biochemical and physiological characteristics and host range. Crouch *et al*. (2009b) demonstrated that the falcate-spored, grass-associated *Colletotrichum* species could be distinguished by a combination of molecular data, appressorial morphology and host range. However, for most *Colletotrichum* species host range has not been determined and available information should be considered with caution, as it is often based on wrongly identified species (Damm *et al*., 2009).

Molecular phylogeny

Due to the inadequacies and plasticity of morphological characters, nucleic acid sequence analysis has been regarded as more reliable for *Colletotrichum* classification (Sutton, 1992; Cannon *et al*., 2000; Crouch *et al*., 2009a,b; Damm *et al*., 2009; Prihastuti *et al*., 2009). A major drawback in the reliance on a small proportion of the genome to understand phylogenetic relationships amongst *Colletotrichum* strains has been the risk of recreating gene trees rather than species trees (Cannon *et al*., 2000). Thus, multi-gene phylogenetics are employed to systematically characterise *Colletotrichum* species relationships and to serve as a base for species diagnosis (Crouch *et al*., 2006, 2009b; Farr *et al*., 2006; Damm *et al*., 2009; Prihastuti *et al*., 2009). Prihastuti *et al*. (2009) used six genes, the nuclear rDNA internal transcribed spacer (ITS) region, partial Actin (ACT), β-tubulin (TUB2), Calmodulin (CAL), Glutamine synthetase (GS) and Glyceraldehyde 3-phosphate dehydrogenase (GPDH) to study a few closely related *Colletotrichum* species (*C. gloeosporioides sensu lato*) and established that species relationships could be well resolved. Multi-gene phylogenies were also successfully applied to resolve the relationships among *Colletotrichum* species with curved conidia from graminicolous and from herbaceous hosts (Crouch *et al*., 2009b; Damm *et al*., 2009). Multi-gene phylogenetics is an accurate and reliable way for the diagnosis of *Colletotrichum* species, but is neither very efficient nor economical. It is currently impractical to apply multiple gene phylogenetics to every *Colletotrichum* species, as different research groups use different gene regions. An international collaborative effort is essential in order to standardise research being carried out on the genus. Hyde *et al*. (2009) listed all the multi-gene sequences derived from the type or epitype cultures of *Colletotrichum*. This provides an excellent platform for data analysis that aims to study the natural relationships among species.

The ITS region is the most widely sequenced region but there are some concerns as to whether ITS sequence data can provide adequate resolution to determine and differentiate *Colletotrichum* species. Crouch *et al*. (2009d) have revealed a high error rate and frequency of misidentification (86%) based on ITS sequence similarity comparison within the *C. graminicola* species complex. ITS sequence data in the public domain can cause considerable confusion to the end user; sequence data were often entered under an incorrect specific name and which may comprise several cryptic species. Identical sequence data has regularly been entered under different names.

We analysed 343 ITS sequences named "*C. gloeosporioides*" (accessed on 6 Sept 2009) and found that >86% had considerable evolutionary divergence from the type specimen of *C. gloeosporioides* (Cannon *et al*., 2008), and most likely represent other *Colletotrichum* species. It is paramount that sequence data generated from type specimens are used in species similarity comparisons and phylogenetic analysis. Due to ease of acquisition and an extensive library of existing sequences, the ITS region is still useful in some cases for reconstruction of interspecific relationships, although it is not an ideal marker for inferring infraspecific relationships. Currently, ITS is also the only gene region that is available from all the ex-type or ex-epitype cultures of *Colletotrichum* species (Table 1). We provide a phylogenetic tree based on ITS sequence data, which includes sequences originated from 42 ex-type or ex-epitype cultures, in order to provide a backbone tree for further diagnosis in *Colletotrichum* (Fig. 1). Taxon names, GenBank accession numbers, culture collection numbers, hosts and origins of the strains are provided in Table 1. The analysis was conducted following the methodology outlined by Cai *et al*. (2008, 2009). The backbone tree can be used to provide a rough, quick identification guide to *Colletotrichum* species.

Colletotrichum systematics should utilise a multiple-gene phylogeny and a study of type specimens to establish correlations between the genotype and phenotype. The phenotype should be expanded to a polyphasic sense (morphology, physiology, pathogenicity, infection processes, cultural characteristics and secondary metabolites) (Frisvad, 2004; Samson and Varga, 2007). The genotypes in *Colletotrichum* appear to correlate with a combination of characters but not with a single character. These criteria are clearly demonstrated by Crouch *et al*. (2009b), Damm *et al*. (2009) and Prihastuti *et al*. (2009). Crouch *et al*. (2009b) correlated the well-defined phylogenetic groupings with combined characters of appressoria and host ranges. Prihastuti *et al*. (2009) established new taxa in the *C. gloeosporioides* complex that form distinct phylogenetic lineages with distinct morphological, cultural and physiological characteristics. Taylor *et al*. (2000) suggested that a genealogical concordance method should be used to recognise a phylogenetic species, with three criteria for each lineage, i.e. monophyletic, statistically supported and genealogically concordant (i.e. no conflict among the single gene tree). Our proposal is broader as we also emphasize the importance of correlation with phenotypic characters, so as to develop species concepts in a systematic, biological sense.

Physiology, carbon source utilization and growth rate

Patterns of carbohydrate utilization have been used to resolve the classification of *Penicillium* species (Bridge, 1985) and this has also been employed to differentiate *Colletotrichum* species (Waller *et al*., 1993; Prihastuti *et al*., 2009). *Colletotrichum kahawae* Waller & Bridge is an economically important pathogen causing coffee berry disease in Africa, which could be distinguished from other *Colletotrichum* species on coffee by its inability to utilize citrate or tartrate as a sole carbon source (Waller *et al*., 1993; Prihastuti *et al*., 2009). There have been considerable controversies as to whether *Colletotrichum kahawae* should be a valid species or a sub-population of *C. gloeosporioides*, as *C. kahawae* can only be distinguished from *C. gloeosporioides* and other close relatives by biochemical and physiological characters (Correll *et al*., 2000; Cannon *et al*., 2000). Recent phylogenetic analysis using multi-gene data showed that *C. kahawae* is genetically distinct from other close related species in the complex (Prihastuti *et al*., 2009). This serves as a good demonstration for correlation between genotypes and phenotypes.

Relative growth rates in culture is a useful criterion for differentiating some *Aspergillus* and *Penicillium* species (Frisvad, 2004; Frisvad *et al*., 2007; Samson and Varga, 2007) and this character has also been employed in delimitating *Colletotrichum* species. For example, *Colletotrichum acutatum* grows significantly slower than *C. gloeosporioides* and can be distinguished by growth rate (Sutton, 1992). Than *et al*. (2008b) and Prihastuti *et al*. (2009) showed a good correlation between growth rate in culture and multi-gene phylogeny in species causing chilli anthracnose and coffee berry disease in

Table 1. rDNA ITS sequences used in the phylogenetic analysis.

Species	GenBank accession numbers	Culture collection No.	Host	Origins	Culture derived from	References of the sequences
C. acutatum	AF411701	IMI 117619	*Carica papaya*	Australia	Paratype	Vinnere *et al.*, 2002
C. acutatum	FJ788417	IMI 117620	*Carica papaya*	Australia	Paratype	Weir and Johnston, unpublished
C. anthrisci	GU227845	CBS 125334	*Anthriscus sylvestris*	Netherlands	Holotype	Damm *et al.*, 2009
C. anthrisci	GU227846	CBS 125335	*Anthriscus sylvestris*	Netherlands		Damm *et al.*, 2009
C. asianum	FJ972612	BPDI4	*Coffea arabica*	Thailand	Holotype	Prihastuti *et al.*, 2009
C. asianum	FJ972605	BMLI3	*Coffea arabica*	Thailand		Prihastuti *et al.*, 2009
C. axonopodi	EU554086	IMI 279189	*Axonopus affinis*	Australia	Holotype	Crouch *et al.*, 2009b
C. boninense	AB051400	MAFF 305972	*Crinum asiaticum* var. *sinicum*	Japan	Holotype	Moriwaki *et al.*, 2003
C. boninense	AB051403	MAFF 306094	*Crinum asiaticum* var. *sincium*	Japan	Paratype	Moriwaki *et al.*, 2003
C. cereale	DQ126177	KS20BIG			Epitype	J.A. Crouch, unpublished
C. cereale	DQ126203	PA50183				J.A. Crouch, unpublished
C. chlorophyti	GU227894	IMI 103806	*Chlorophytum* sp.	India	Holotype	Damm *et al.*, 2009
C. chlorophyti	GU227895	CBS 142.79	*Stylosanthes hamata*	Australia		Damm *et al.*, 2009
C. cliviae	GQ485607	CSSK4	*Clivia miniata*	China	Holotype	Yang *et al.*, 2009
C. coccodes		CBS 164.49	*Solanum tuberosum*	Netherlands	Epitype	L. Cai, unpublished
C. coccodes		CBS 369.75	*Solanum tuberosum*	Netherlands		L. Cai, unpublished
C. curcumae	GU227893	IMI 288937	*Curcuma longa*	India	Epitype	Damm *et al.*, 2009
C. dematium	GU227819	CBS 125.25	*Eryngium campestre*	France	Epitype	Damm *et al.*, 2009
C. dematium	GU227820	CBS 125340	*Apiaceae*	Czech Republic		Damm *et al.*, 2009
C. dracaenophilum	DQ286209	CBS 118119	*Dracaena* sp.	China	Holotype	Farr *et al.*, 2006
C. dracaenophilum	EU003533	CBS 121453	*Dracaena sanderiana*	Bulgaria		Bobey *et al.*, 2008
C. eleusines	EU554131	MAFF 511155		Japan	Epitype	Crouch *et al.*, 2009b
C. falcatum	FJ972606		*Saccharum officinarum*	Indonesia	Epitype	Prihastuti *et al.*, 2009
C. fioriniae	EF464594	EHS58	*Fiorinia externa*	USA	Holotype	Shivas and Tan, 2009
C. fioriniae	EF464593	EHS48	*Fiorinia externa*	USA	Paratype	Shivas and Tan, 2009
C. fructi	GU227844	CBS 346.37	*Malus sylvestris*	USA	Epitype	Damm *et al.*, 2009
C. fructicola	FJ972603	BPDI16	*Coffea arabica*	Thailand	Holotype	Prihastuti *et al.*, 2009
C. fructicola	FJ972611	BPDI12	*Coffea arabica*	Thailand		Prihastuti *et al.*, 2009
C. gloeosporioides	EU371022	IMI 356878	*Citrus sinensis*	Italy	Epitype	Cannon *et al.*, 2008
C. gloeosporioides	FJ972609	CBS 953.97	*Citrus sinensis*	Italy		Prihastuti *et al.*, 2009
C. hanaui	EU554124	MAFF 511014	*Digitaria ciliaris*	Japan		Crouch *et al.*, 2009b
C. hanaui	EU554101	MAFF 305404	*Digitaria ciliaris*	Japan	Holotype	Crouch *et al.*, 2009b
C. hippeastri	GQ485599	CSSG1	*Hippeastrum vittatum*	China	Holotype	Yang *et al.*, 2009
C. hippeastri	GQ485598	CSSG2	*Hippeastrum vittatum*	China		Yang *et al.*, 2009

Table 1 (continued). rDNA ITS sequences used in the phylogenetic analysis.

Species	GenBank accession numbers	Culture collection No.	Host	Origins	Culture derived from	References of the sequences
C. horii	GQ329690	ICMP 10492	*Diospyros kaki*	Japan	Neotype	Weir and Johnston, in press
C. horii	AY791890	TSG002	*Diospyros kaki*	China		J.Z. Zhang, unpublished
C. hymenocallidis	GQ485600	CSSN2	*Hymenocallis americana*	China	Holotype	Yang *et al.*, 2009
C. hymenocallidis	GQ485601	CSSN3	*Hymenocallis americana*	China		Yang *et al.*, 2009
C. jacksonii	EU554108	MAFF 305460	*Echinochloa esculenta*	Japan	Holotype	Crouch *et al.*, 2009b
C. jacksonii	EU554130	MAFF 511152	*Echinochloa esculenta*	Japan		Crouch *et al.*, 2009b
C. kahawae	FJ972608	IMI 319418	*Coffea arabica*	Kenya	Holotype	Prihastuti *et al.*, 2009
C. kahawae	FJ972607	IMI 363578	*Coffea arabica*	Kenya		Prihastuti *et al.*, 2009
C. lineola	GU227829	CBS 125337	*Apiaceae*	Czech Republic	Epitype	Damm *et al.*, 2009
C. lineola	GU227830	CBS 125339	*Apiaceae*	Czech Republic		Damm *et al.*, 2009
C. lupine	AJ301948	BBA 70884	*Lupinus albus*	Ukraine	Neotype	Nirenberg *et al.*, 2002
C. lupine	AJ301930	BBA 63879	*Lupinus mutabilis*	Bolivia		Nirenberg *et al.*, 2002
C. lupine var. *setosum*	AJ301923	BBA 70352	*Lupinus albus*	Germany	Holotype	Nirenberg *et al.*, 2002
C. lupine var. *setosum*	AJ301933	BBA 70358	*Lupinus albus*			Nirenberg *et al.*, 2002
C. miscanthi	EU554121	MAFF 510857	*Miscanthus sinensis*	Japan	Holotype	Crouch *et al.*, 2009b
C. navitas	GQ919067	CBS 125086	*Panicum virgatum*	USA	Holotype	Crouch *et al.*, 2009c
C. navitas	GQ919068	9032d	*Panicum virgatum*	USA		Crouch *et al.*, 2009c
C. nicholsonii	EU554126	MAFF 511115	*Paspalum dilatatum*	Japan	Holotype	Crouch *et al.*, 2009b
C. nicholsonii	EU554122	MAFF 510916	*Paspalum dilatatum*	Japan		Crouch *et al.*, 2009b
C. paspali	EU554100	MAFF 305403	*Paspalum notatum*	Japan	Holotype	Crouch *et al.*, 2009b
C. paspali	EU554123	MAFF 510000	*Paspalum notatum*	Japan		Crouch *et al.*, 2009b
C. rusci	GU227818	CBS 119206	*Ruscus* sp.	Italy	Holotype	Damm *et al.*, 2009
C. siamense	FJ972613	BPDI2	*Coffea arabica*	Thailand	Holotype	Prihastuti *et al.*, 2009
C. siamense	FJ972614	BMLI15	*Coffea arabica*	Thailand		Prihastuti *et al.*, 2009
C. simmondsii	FJ972601	BRIP 28519	*Carica papaya*	Australia	Holotype	Prihastuti *et al.*, 2009
C. simmondsii	FJ972610	CBS 294.67	*Carica papaya*	Australia		Prihastuti *et al.*, 2009
C. spaethianum	GU227807	CBS 167.49	*Funkia sieboldiana*	Germany	Epitype	Damm *et al.*, 2009
C. spaethianum	GU227808	CBS 100063	*Lilium* sp.	South Korea		Damm *et al.*, 2009
C. sublineolum	DQ003114	S3001			Epitype	J.A. Crouch, unpublished
C. sublineolum	DQ195716	BRIP 1402				J.A. Crouch, unpublished
C. truncatum	GU227862	CBS 151.35	*Phaseolus lunatus*	USA	Epitype	Damm *et al.*, 2009
C. truncatum	GU227877	CBS 120709	*Capsicum frutescens*	India		Damm *et al.*, 2009
C. verruculosum	GU227806	IMI 45525	*Crotalaria juncea*	Zimbabwe	Holotype	Damm *et al.*, 2009
C. yunnanense	EF369490	AS 3.9617	*Buxus* sp.	China	Holotype	Liu *et al.*, 2007
C. yunnanense	EF369491	AS 3.9616	*Buxus* sp.	China		Liu *et al.*, 2007

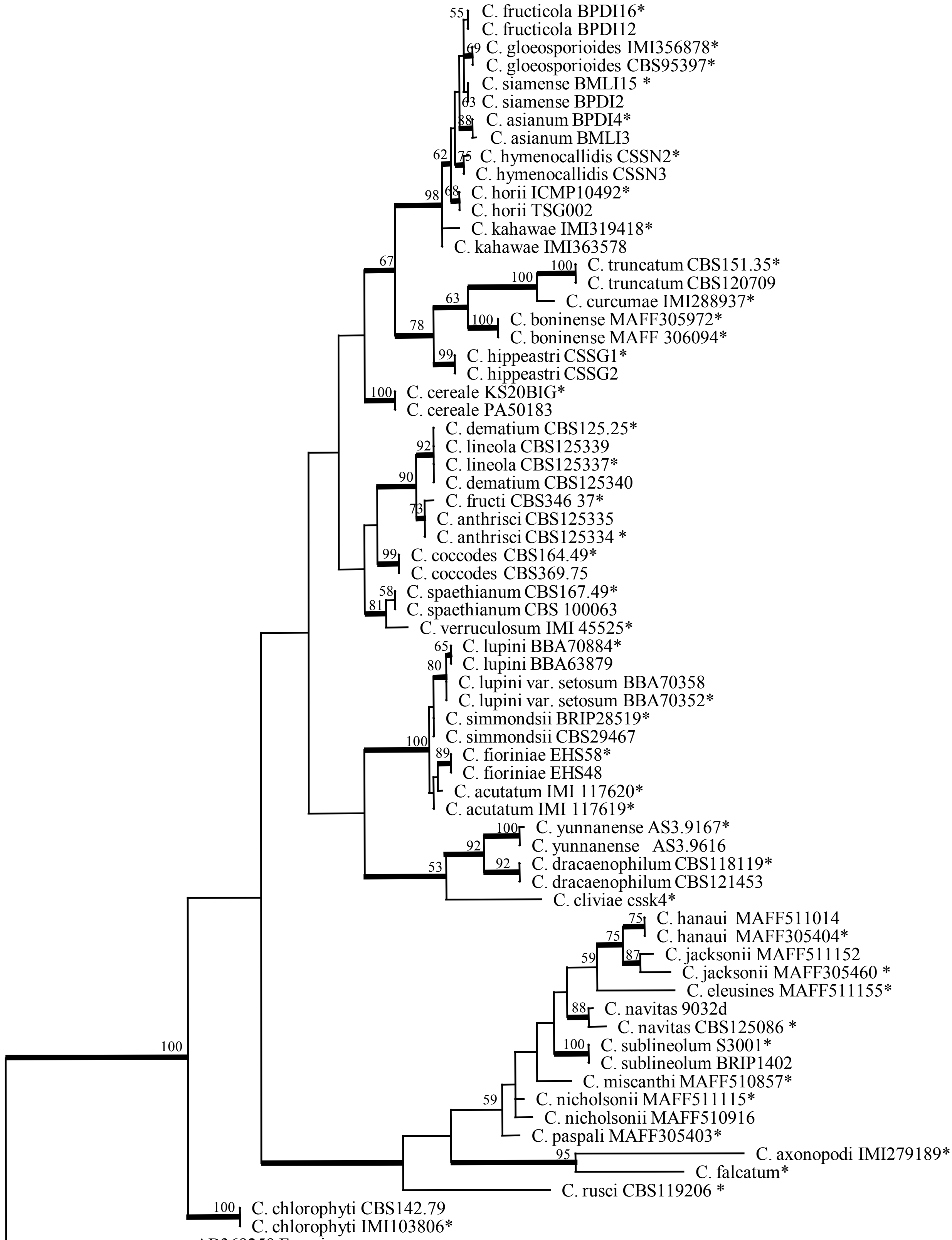

Fig. 1. Phylogram generated from parsimony analysis based on rDNA ITS sequence data. Data was analysed with random sequence addition, unweighted parsimony and by treating gaps as missing data. Bootstrap values ≥ 50% are shown above or below branches. Thickened branches indicate Bayesian posterior probabilities ≥95%. The tree is rooted with *Fusarium oxysporum.* (*Cultures derived from type specimens).

Thailand. *Colletotrichum asianum* could easily be distinguished from *C. fructicola* and *C. siamense* (all from coffee) by its much slower growth rate (Prihastuti *et al*., 2009).

The usefulness of physiological characters in differentiating closely related species in *Colletotrichum* has been well demonstrated in a few species. Unfortunately, such physiological characterizations have not been made for most *Colletotrichum* species. In this paper, we suggest that the carbohydrate utilization and relative growth rate should be used as one of the polyphasic characters to study *Colletotrichum*, especially for those morphologically indistinguishable species that bear phylogenetic distinctiveness.

Pathogenicity testing

It is important to establish whether a particular *Colletotrichum* species is host-specific or has a wide host range. Host-specific taxa may have a limited distribution, which may have important biosecurity implications. If the taxon has a wide host range the species is likely to be cosmopolitan and possibly an opportunistic pathogen. Host range studies may provide data useful in classification and future delimitation of species. Koch's postulates have been completed to confirm the pathogenicity of various *Colletotrichum* isolates. For example, Vinnere *et al*. (2002) showed that *Rhododendron* anthracnose was caused by *C. acutatum*; Lee *et al*. (2005) re-infected *Euonymus* leaves with *C. boninense* in moist chamber pathogenicity tests; Tomioka *et al*. (2008) showed that a species referred to as *C. dematium* (but see Damm *et al*., 2009) was the causal agent of severe spotting, blight and drop of leaves of *Polygonatum falcatum*. Descriptions of new species sometimes include data on pathogenicity testing (Nakamura *et al*., 2006; Moriwaki and Tsukiboshi 2009; Yang *et al.,* 2009), although this is not usually the case (e.g. Farr *et al*., 2006). Crouch *et al*. (2009c) confirmed that a new species, *C. navitas*, caused anthracnose of *Panicum virgatum*. Much of the pathogenicity work that has been carried out needs repeating with verified *Colletotrichum* species. For example, pathogenicity tests using *C. acutatum, C. capsici* and *C. gloeosporioides* isolates from chili were carried out on susceptible and resistant varieties of chili (Than *et al.,* 2008b) but names of the *Colletotrichum* species used need to be re-examined in light of a re-assessment of some of these *Colletotrichum* species (Shivas and Tan, 2009). Papers epitypifying plant pathogenic taxa should ideally include pathogenicity testing, but this has not usually been done (e.g. Shenoy *et al*., 2007; Cannon *et al*., 2008; Than *et al*., 2008a; Crouch *et al*., 2009b; Weir and Johnston, in press).

Other possible methods

Cross mating experiments

A cross mating test is an in vitro experiment employed to recognise fungal species based on the biological species concept (Mayr, 1940; Taylor *et al*., 2000). In biological species recognition, groups of mating compatible individuals are regarded as the same species (Taylor *et al*., 2000). There are major obstacles that limit the application of this theory to fungi including: 1) many fungi are asexual (they may have permanently lost the ability to reproduce sexually) and do not produce ascospores (Reynolds, 1993); 2) some species are able to produce ascospores without a partner (homothallic), e.g. *C. falcatum* and *C. graminicola* (von Arx and Müller, 1954; Politis, 1975; Vaillancourt and Hanaui, 1991); 3) failure of some heterothallic fungi to mate in artificial cultivation; and 4) there is much evidence that groups of genetically isolated fungi may retain the ancestral characters of interbreeding. A common result is that mating tests may define species that encompass more than one genetically and phylogenetically isolated groups (Perkins and Turner, 1988; Chase and Ullrich, 1990; Vilgalys and Sun, 1994; Petersen and Hughes, 1999).

There have been few studies using cross mating tests to distinguish between *Colletotrichum* species. Guerber *et al*. (2003) tested isolates of *Colletotrichum acutatum sensu lato* and concluded that two phylogenetically isolated clades have retained the ability to mate. Genetic isolation appears to have occurred before reproductive isolations in the *C. acutatum* complex. In the case of the *Colletotrichum gloeosporioides* complex, sexual compatibility had been reported to be limited to individuals that share the same host range (Brasier, 1987).

Cisar *et al*. (1994) discovered that some isolates of *C. gloeosporioides* that are pathogenic to distantly related hosts are also sexually compatible. This result is in agreement with studies on *C. acuatum*, i.e. genetically isolated strains may retain the ability to mate. Similar findings have been reported for *Fusarium* and other fungi (Donoghue, 1985; Taylor *et al*., 2000; Leslie *et al*., 2001; Amata *et al*., in press).

It is noteworthy that most *Colletotrichum* species lack a sexual state (Hyde *et al*., 2009), and many of the known teleomorphs, such as those of *C. graminicola* and *C. sublineolum* (Carvajal and Edgerton, 1944; Politis, 1975; Vaillancourt and Hanau, 1992), are characterised based on mating experiments in the laboratory. In this sense, mating experiments are important for a comprehensive understanding of *Colletotrichum* species and should be undertaken in studies on *Colletotrichum*.

Secondary metabolite profiles

There have been numerous publications on secondary metabolite production by *Colletotrichum* species and the chemical structures of some of these metabolites have been elucidated. However there have been few attempts to use metabolite profiling as a source of markers for identification and classification purposes (O'Connell *et al*., 1998; Abang *et al*., 2009), or to integrate chemotaxonomy within a polyphasic framework for resolving natural relationships of taxa within *Colletotrichum*. Secondary metabolite production has been extensively studied in putative strains of *C. capsici*, *C. dematium, C. fragariae*, *C. gloeosporioides*, *C. lagenarium*, *C. nicotianae* and *C. truncatum* but was not linked to taxonomy (García-Pajón and Collado, 2003). This is in distinct contrast to *Alternaria, Annulohypoxylon, Aspergillus*, *Fusarium*, *Hypoxylon, Penicillium*, *Stachybotrys*, *Stemphylium*, and *Trichoderma* species, where secondary metabolites have been found to be valuable taxonomic markers (Stadler *et al*., 2004; Frisvad *et al*., 2007, 2008; Anderson *et al*., 2008;).

One of the few studies of secondary metabolites as taxonomic markers for distinguishing *Colletotrichum* spp. involved the use of lectins - proteins or glycoproteins that contain binding sites complementary in shape to particular monosaccharides or oligosaccharides (O'Connell *et al*., 1998). Although lectin cytochemistry proved to be valuable as a chemotaxonomic tool, the technique is limited by the relatively small number of sugars that are recognised by lectins. A further limitation of this approach is the difficulty to interpret assay results when lectins show affinity for more than one sugar or when binding involves non-specific hydrophobic or ionic interactions (O'Connell *et al*., 1998). O'Connell *et al*. (1998) proposed the use of monoclonal antibodies (MAb) in studies of *Colletotrichum* identification and chemotaxonomy because they offer a much wider range of binding sites than lectins. Although antibodies have been used in identification of species such as *C. gloeosporioides* (Peters *et al*., 1998) and in taxonomic studies of zoosporic fungi (Hardham *et al*., 1991), their wider applicability in resolving *Colletotrichum* systematics remains to be investigated.

Morphotype, virulence phenotype, phylotype and chemotype were recently used in a polyphasic approach to clarify the taxonomic status of *Colletotrichum* isolates associated with anthracnose disease of yam (*Dioscorea* spp.) (Abang *et al*., 2009). Four morphotypes of *C. gloeosporioides sensu lato* were recognised associated with foliar anthracnose of yam: slow growing grey (SGG); fast growing salmon (FGS); fast-growing grey (FGG); and fast growing olive (FGO). The FGG morphotype showed a greater divergence from the other three morphotypes based on ITS sequence data. Secondary metabolite profiles in high performance TLC (HPTLC) and high performance liquid chromatography (HPLC) showed that the pathogenic SGG and FGS forms had a chemotype (A or B) that was distinct from the non-pathogenic FGG form (chemotype C). A highly phytotoxic HPLC fraction was detected in virulent FGS and SGG strains, but was not detected in the FGG strains. It was not possible to distinguish the pathogenic FGS from SGG forms of *Colletotrichum* based on their ITS-based phylotype; however, they could be clearly distinguished based on their combined ITS and metabolite profiles (Abang *et al*., 2009), which corroborated a previous finding that these strains represented two genetically distinct populations of *C. gloeosporioides* *sensu lato* on yam (Abang *et al*., 2005). The presence of outliers stresses the necessity to have a large number of correctly identified strains of the same taxon for robust chemotaxonomic analyses (Anderson *et al*., 2008).

The chemotaxonomic approach used to elucidate the taxonomic status of *Colletotrichum* from yam could be applied to resolve the systematics of the genus as a whole. Frisad *et al*. (2008) noted that, "the use of secondary metabolite profiling seems to be of greatest value in Ascomycetes…" which should motivate those planning to apply this tool to *Glomerella*/*Colletotrichum* taxonomy. There are important methodological considerations that should be borne in mind in chemotaxonomic studies, many of which have been discussed by Frivad *et al*. (2008) and Anderson *et al*. (2008). For instance, fungal cultures used in comparative chemotaxonomic analysis should be grown on the same medium, incubated together at the same temperature, and extracted at the same time, to ensure that differences reflected fungal diversity and not environmental conditions (Frisad *et al*., 2008). HPLC methods also need to be standardized and the lack of standards for certain known metabolites makes it difficult to identify many peaks in HPLC-DAD chromatograms.

A new approach to *Colletotrichum* systematics, which utilizes metabolite profiling (thus bringing functional characters to the fore), may help provide a better understanding of species relationships in this genus. The correct identification of a species and its strains using ITS and other sequence data, is, however, essential before such studies can take place. Research in chemotaxonomy in *Colletotrichum* is at an early stage and recommendations are not made here.

Infraspecific taxonomy

Infraspecific groups within *Colletotrichum* species are poorly understood and have been mostly avoided in the paper on current names (Hyde *et al*., 2009). The current Code of Botanical Nomenclature provides a few formal infraspecific categories: subspecies, variety and form. Categories such as forma specialis and pathotype have also been used by plant pathologists for infraspecific groups with distinct host specializations or behaviours (Cannon, 2000).

The use of subspecies, varieties and forms within *Colletotrichum* have been variously used with 33 introductions between 1940 and 2000. The fact that forma species or varieties were intermittently used appear to have depended as much on individual authors as on any rules and until a clear understanding of what constitutes a *Colletotrichum* species it may be unwise to consider subspecies, forms and varieties any further. From a pathology view point there has been more recent use of forma specialis and pathotype, with pathotypes being most frequently used in the genus (Lubbe *et al*., 2004; Suman *et al*., 2005; Moore *et al*., 2008).

For true pathotype differences there must be a qualitative or phenotypic difference in infection (virulence) between isolates on a set of differential genotypes (Taylor and Ford, 2007). A quantitative difference in severity of infection based on lesion size is simply a reflection of the variation of aggressiveness of isolates and does not constitute a true pathotype difference (Taylor and Ford, 2007).

Identification of pathotypes of *Colletotrichum* species has been based on both qualitative differences in infection, e.g. *C. trifolii* in lucerne (Mackie *et al*., 2003); *C. lindemuthianum* in bean (Gonzalez-Chavira *et al*., 2004); *C. acutatum* in citrus (You *et al*., 2007); *C. sublineolum* in sorghum (Moore *et al*., 2008); *C. capsici* in chili pepper (Montri *et al*., 2009); *C. acutatum* and *C. gloeosporioides* in chili pepper (Monkolporn *et al*., 2010) and quantitative differences in severity (*C. capsici* in chili pepper, Sharma *et al*. 2005; *C. falcatum* in sugarcane, Suman *et al*., 2005) between isolates on specific host genotypes.

There have been some reports of races of *Colletotrichum* species; however, races of a fungal pathogen only occur where differences in isolates are determined by differences in virulence genes and corresponding host resistance genes, i.e. a gene for gene relationship as occurs in rust diseases. Mackie *et al*. (2007) linked races 1, 2 and 4 of *C. trifolii* to quantitative trait loci (QTL) conferring resistance to *C. trifolii* in lucerne. However, since the relationship between specific genes for avirulence in the pathogen and resistance genes in the host has not been established, these variations in the pathogen should be referred to as pathotypes as described by Irwin *et al*. (2006).

The identification of pathotypes is not only important as a taxonomic tool at the infraspecific level of a *Colletotrichum* species, but has implications for plant breeders trying to develop new improved genotypes with durable resistance to a pathogen. Pathotypic differences actually help to relate the infraspecific taxonomy back to the biological interaction of the pathogen to specific genotypes of a host.

The future

The identification of *Colletotrichum* species needs to be simple and quick as it has important implications for biosecurity, disease control and plant breeding. There is now a major effort to establish species concepts and formalise names in current use. It is unlikely that morphology will solve many problems in species delineation, but a polyphasic approach can be used to establish species boundaries, for epitypification of existing names and for the introduction of new species. Ideally, a single gene will be found that can be used for barcode-like identification of species in a quick and simple way.

Searching a suitable gene for barcoding

DNA barcoding aims to establish an accurate, rapid, cost-effective and universally accessible identification system for organisms by using short and standardised segments of the genome (Herbert *et al*., 2003, 2004; Summerbell *et al*., 2005). It is particularly important to establish a DNA barcoding system for *Colletotrichum* species, as they have very simple morphologies and the current taxonomy is very confused. The success of *Colletotrichum* barcoding relies on highly reliable sequences from the type or verified strains and the selection of most appropriate of gene(s).

The *Colletotrichum gloeosporioides* species complex contains taxa with significant biological, morphological, and genetic diversity (Sutton, 1992; Hyde *et al*., 2009). Some of these taxa have been named as separate species e.g. *C. fragariae* (Johnston *et al.,* 2008). ITS region has a poor ability to discriminate clades within this group, hence a more appropriate biomarker is required for use in barcoding. The ideal locus for use in barcoding has low heterogeneity within species, yet enough variation to allow maximum separation of different species. The first requirement allows strains of the same species to be easily grouped and identified. The second requirement ensures that misidentifications are minimised. It is also important, that the gene can be easily sequenced for all species and does not require specific primers for each group (Seifert, 2009; Gilmore *et al*., 2009; Chen *et al*., 2009).

We used a diverse set of 64 *Colletotrichum gloeosporioides sensu lato* isolates (and two *C. boninense* strains as outgroups) previously analysed by Johnston *et al*. (2008). Each isolate was sequenced with six genes: ITS, GPDH (glycerol-3-phosphate dehydrogenase), CAL (calmodulin), ACT (actin), CHS (chitin synthase), and EF1α (elongation factor 1α). The 64 taxa were assigned to five groups on the basis of a multigene phylogeny (Johnston *et al*., 2008). Two groups represented named species (*C. fragariae* and *C. horii*) and one group represented *Colletotrichum gloeosporioides sensu stricto*. The remaining two groups A and B (*sensu* Johnston and Jones, 1997) consisted of diverse mostly unnamed isolates, although *C. musae* is in Group A, and *C. kahawae* is in Group B. A similarity matrix was generated for each gene, and results were analysed in TaxonGap 2.4.1 (Slabbinck *et al*., 2008).

The results (Fig. 2) graphically summarise the heterogeneity within each of the assigned groups (light grey bar), and the separability of species (dark grey bar). Separability represents the ability of the gene to distinguish between groups, the minimum separability of GPDH, CAL, ACT, and EF1α is good at 12.2, 11.7, 9.5, and 9.9, respectively. In contrast ITS and CHS have low minimum separability values of 3.9 and 0.3, respectively.

The heterogeneity values (intra-species variation) ideally should be low, certainly less than the respective separability value. In most cases this is true, with the notable exception of EF1α in Groups A, B, *sensu stricto*, and *C. boninense*. This is likely the result of the high variability of introns in EF1α sequences of

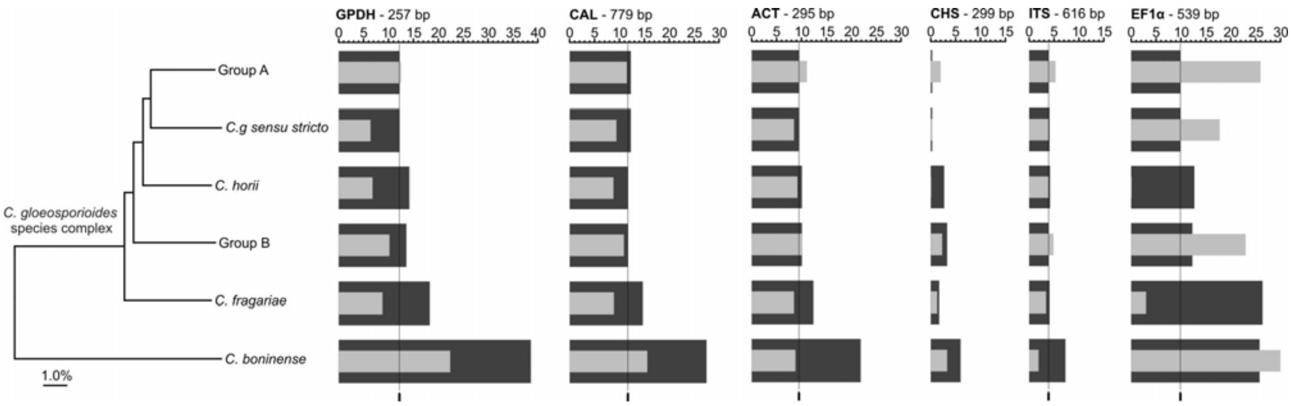

Fig. 2. Comparison of intra- and inter-species variation for six potential barcode genes. Species / groups were ordered according to their position in a maximum likelihood phylogenetic tree generated from concatenated sequences of representative strains. Separability (inter-species variation) is presented as dark grey bars, and heterogeneity (intra-species variation) is presented as light grey bars. The vertical black line denotes the smallest separability recorded. *C. boninense* was used as an outgroup.

Colletotrichum species, which is likely to be more useful in population level studies, rather than systematics or barcoding. Across all the genes the heterogeneity values for Groups A and B are high, this may imply that these groups consist of several different taxa, and should be subdivided further. It can be concluded from this analysis that within *Colletotrichum gloeosporioides sensu lato* GPDH, CAL, and ACT are good candidates for barcodes; whilst ITS, CHS, and EF1α are poor candidates.

A similar set of six genes (ITS, ACT, CHS, GPDH, histone 3, beta-tubulin) was used by Damm *et al.* (2009) for studying *Colletotrichum* species with curved conidia from herbaceous hosts, which included six different clades. The separability of the species was best with both GPDH and histone 3 genes, which are superior to ITS, ACT, CHS and beta-tubulin.

The most appropriate gene for barcoding *Colletotrichum* must be selected in the process of species delimitation, since we can only decide for one or more barcoding gene(s) when we know which taxa need to be distinguished by it. A good approach would be to test and establish a selection of loci used by the different groups involved in *Colletotrichum* systematics in order to work towards the best barcoding gene.

Detailed protocols for studying *Colletotrichum* species

Isolation

Endophytes and pathogens without visible sporulation: Plant tissues are cut into small pieces, surface sterilized by dipping in 1% sodium hypochlorite for 1 minute, in 70% ethanol for 1 minute and rinsed three times with sterilized water and finally dried on sterilized tissue paper. The plant tissues are then placed onto potato dextrose agar (PDA) containing 100 μg/ml streptomycin and 50 μg/ml tetracycline to allow fungal growth. The mycelia growing from pieces of plant tissues are transferred onto a new PDA plate for morphological or molecular study (Than *et al.*, 2008b).

Pathogens or epiphytes with visible sporulation: A single spore isolation technique should be applied to plant tissues where spore masses are formed. Spore masses are transferred with a sterilized wire loop or fine forceps and suspended in sterilized water. The spore suspension is diluted to a reasonable concentration and spread onto the surface of PDA agar, followed by incubation overnight at 25°C. Single germinated spores are picked up with a sterilised needle and transferred onto new PDA plate for further study (Goh *et al.*, 1999).

Morphological studies

There is a need to standardise incubation parameters for *Colletotrichum* species as it is difficult or impossible to compare the morphology of species that have been grown under different conditions. For inoculation, it is suggested to aseptically punch and transfer a mycelial disc (about 4 mm diam.) from the actively growing edge of a 5 day old single conidium derived culture onto PDA. Cultures are incubated at 20, 25 and 30°C under constant fluorescent light. We do not suggest Sutton's protocol as we think the switch of ultra violet irradiation and dark is not always practical and may bring in many more factors that might impact the growth of fungus. Three replicate cultures of each isolate should be investigated. After 7 days, conidial size and shape from >25 conidia should be measured and recorded, while colony characters can be recorded and photographed (Than *et al*., 2008a). Observation and measurements (e.g. conidial size, appressoria size and conidiogenous cells) should be made in water mounts.

Appressoria should be produced using a slide culture technique, in which 10 mm^2 plugs of PDA are placed in an empty Petri dish. The edge of the agar is inoculated with spores taken from a sporulating culture and a sterile cover slip is placed over the inoculated agar (Johnston and Jones, 1997). After 7 days, the shape and size of the appressoria formed on the underside of the cover slip could be recorded (Fig. 3).

Multi-locus phylogeny

Multi-locus phylogeny is a powerful tool to diagnose *Colletotrichum* species and has been widely employed (Johnston *et al*., 2008; Crouch *et al*., 2009b; Prihastuti *et al*., 2009; Yang *et al*., 2009). Unfortunately, dif-ferent research groups have been utilizing different gene regions. For example Crouch *et al*. (2009b) used ITS, Apn2, Sod2 and Mat1-2 gene regions; Prihastuti *et al*. (2009) used ITS, CAL, GS, GPDH, ACT and TUB2 gene regions; Johnston *et al*. (2008) employed additional gene regions such as EF1α and CHS. To facilitate future study and comprehensive comparison of *Colletotrichum* species, it is important that an agreement should be formed on how many and which genes should be sequenced. An even more important issue is the interpretation of phylogenetic groups in terms of classification. Currently there is no consensus over what constitutes a species, although researchers started to adopt "genealogical concordance" to recognise phylogenetic species (Taylor *et al*., 2000). In this paper we suggest that species rank should be given to well defined phylogenetic lineages that are in conjunction with recognisable phenotypic characters.

Growth rate

Punch and aseptically transfer a mycelial disc (about 4 mm diam.) from the actively growing edge of a 5 day old single conidium derived culture onto PDA. Cultures are incubated at 25°C under constant fluorescent light. Three replicate cultures of each isolate should be investigated. Colony diameter of the culture is measured daily for 7 days. Growth rate can be calculated as the 7 day average of mean daily growth (mm per day). After 7 days, colony size and colour of the conidial masses and zonation should be recorded.

Biochemical tests

Biochemical testing can also be utilized as it has been reported in several studies that biochemical characters, especially the ability to utilize citrate or tartrate as a sole carbon source could be used to differentiate some closely related species (Bridge *et al*., 2008; Prihastuti *et al*., 2009). The biochemical test based on substrate utilization could be assessed in agar plates according to the method of Bridge *et al*. (2008). Utilization of citrate and tartrate as a carbon source are assayed on agar plates. Medium B (contains $NH_3H_2PO_4$ 1.0g/L; KCl 0.2 g/L; $MgSO_4.7H_2O$ 0.2g/L in distilled water) with 1.2% (w/v) agar is supplemented with 1% (w/v) citric acid or ammonium tartrate and 0.005% (w/v) bromocresol purple (Waller *et al*., 1993; Bridge *et al*., 2008). Positive and negative controls containing, respectively, glucose or no additional carbon source are included for each isolate.

All media should be adjusted to pH 4.5 with NaOH or HCl prior to sterilization (Waller *et al*., 1993; Bridge *et al*., 2008). Test media are inoculated with agar plugs (4 mm diam) taken from 5 day old single conidium derived cultures. Utilization is assessed by visual comparison of growth and a rise in the pH of the medium adequate to produce a dark blue to purple colour of bromocresol purple (Waller *et al*., 1993; Bridge *et al*., 2008).

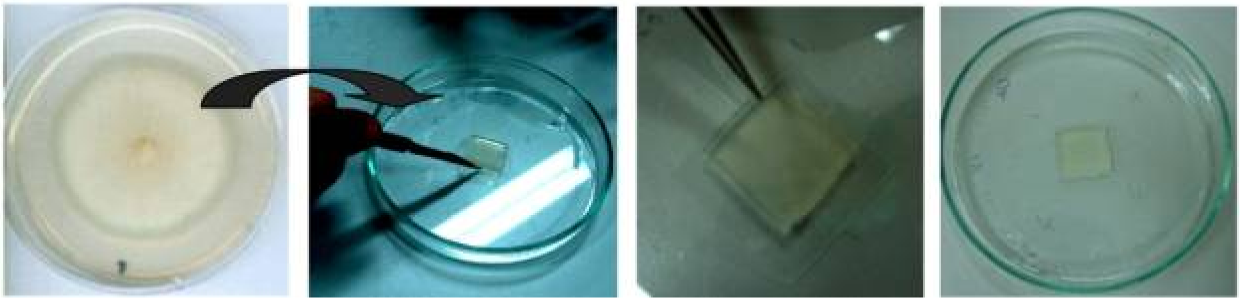

Fig. 3. Slide culture technique to induce the formation of appressoria. A. Sporulating culture used to make spore suspension. B. Spore suspension applied to a 10 mm square of PDA agar. C. Agar square covered by a sterilised cover slip. D. Incubation at 25°C for 7days.

Pathogenicity testing

Preparation of inocula and test hosts

Pure cultures of each isolate are grown on PDA for 7–14 days at 25°C under alternating 12 hour fluorescent light /12 hour dark cycle to induce sporulation (Than *et al.*, 2008b). The conidia are harvested by placing 1–5 ml sterilized distilled water onto the culture, which is then gently swirled and scraped to harvest the conidia. The conidial suspension is filtered through two layers of muslin cloth. The spore density is adjusted to a concentration of 1×10^6 spore/ml using a haemocytometer (Tshering, 2006).

Freshly harvested, untreated, mature but unripe fruits, leaves or other part plants are washed under running tap water for 60 seconds followed by surface sterilization by immersing the fruits in 70% ethanol for 3 minutes, 1% sodium hypochlorite solution for 3 minutes and then rinsing three times in sterilised distilled water for 2 minutes each time and drying with sterile tissue paper and then air drying (Sanders and Korsten, 2003; Montri *et al.*, 2009).

Inoculation

The surface sterilized fruits and leaves are placed in a plastic box that contains sterile tissue paper soaked in sterile distilled water to maintain around 95% relative humidity (Montri *et al.*, 2009). The samples are inoculated using the wound/drop and non-wound /drop inoculation methods (Lin *et al.*, 2002; Kanchana-udomkan *et al.*, 2004; Than *et al.*, 2008b). Inoculation with wound/drop method is accomplished by pin-pricking the fruits or leaf with a sterile needle in the middle portion of fruit or leaf and then placing 6 µl of conidia suspension onto the wound (Freeman and Shabi, 1996; Than *et al.*, 2008b). Control fruits and leaves are inoculated with 6 µl of sterile distilled water. The inoculated samples are incubated in containers at room temperature in normal light regimes for 7–14 days (Figs 4–5) (Than *et al.*, 2008b). The non-wound/drop method involves placing 6 µl of conidial suspension onto the middle of each fruit or leaf (Lin *et al.*, 2002; Kanchana-udomkan *et al.*, 2004).

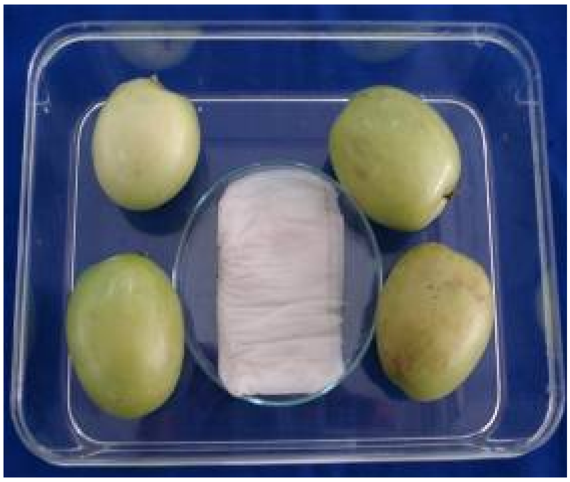

Fig. 4. Incubation of fruits inoculated with spore suspension.

Disease evaluation and data analysis

Evaluation of anthracnose symptoms such as lesion appearance, lesion size, conidia characteristics and the severity of infection is carried out from 7 to 14 days after inoculation (Figs 6–7). Lesion development on fruits and leaves are assessed by the percentage of disease area on each fruit and leaf (lesion area divided by the total fruit/leaf area). Symptoms are evaluated and scored on a 0-9 point scale based on the percentage of the infected area as outlined by Montri *et al.* (2009) (Table 2).

Table 2. Anthracnose severity scores and the symptom description.

Score	Symptom description
0	no infection.
1	1–2% of the fruit area shows necrotic lesion or a larger water soaked lesion surrounding the infection site.
3	>2–5% of the fruit area shows necrotic lesion, acervuli may be present/or water lesion up to 5% of the fruit surface.
5	>5–15% of the fruit area shows necrotic lesion, acervuli present/or water soaked lesion up to 25% of the fruit surface.
7	>15–25% of the fruit area shows necrotic lesion with acervuli
9	>25% of the fruit area shows necrosis, lesion often encircling the fruit, abundant acervuli.

Fig.5. Incubation of leaves inoculated with spore suspension.

It is worth mentioning that artificial inoculations are often conducted on detached or whole plants under extreme conditions and the success of infection appears to be dependent on inoculum density (Bailey and Jeger, 1992), whereas no infection may occur under field conditions (Freeman *et al*., 1998). Artificial host inoculation is, to some extent, not reliable for determining host range or host-specificity, but is an indicator of infection potential (Freeman *et al*., 1998). Some research has shown that pathogenicity tests may not be reliable on detached plant tissues because of suppression of host defense pathways (Liu *et al*., 2007).

Mating test

We suggest the protocol as outlined by Guerber and Correll (2001) and Guerber *et al*. (2003). Modified Czapek-dox agar media (2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$ and 20 g agar, per liter, pH7.8) is used. For inoculation, aseptically punch and transfer a mycelial disc (4 mm diam.) from the actively growing edge of a 5 day old single conidium derived culture to the agar. Two parental isolates are placed opposite to each other and approximately 1 cm from the edge of 9 cm Petri plates. Autoclaved flat woody toothpicks are applied to serve as a substrate for ascomata formation. Three toothpicks are placed on the agar in an “N” arrangement. Plates are incubated at 25ºC under constant cool white fluorescent light. After 20–30 days, the mating plates are examined under a microscope for the presence of ascomata. Successful crosses result in a ridge of mature ascomata containing asci with ascospores being formed at the line of contact. Ascomata are removed from the toothpick using fine forceps and mounted in a drop of sterilized water and crushed under a cover slip. A combination of a qualitative and quantitative rating system is used to score the sexual fertility of each cross. Fertility of cross is scored on a scale of 0–7 (Guerber *et al*., 2003): 0 = no structures; 1 = small sterile structures; 2 = sterile perithecia with beaks, no asci; 3 = sterile perithecia with asci, no ascospores; 4 = asci with a few ascospores; 5 = fertile perithecia with many ascospores, few asci with eight spores; 6 = fertile perithecia with abundant ascospores, many asci with eight spores; 7 = perithecia exuding ascospores from ostiole. Crosses scored 5 or higher are considered fertile. Ascospore viability should be assessed for crosses with a fertility score above 4. Monoascospore cultures are obtained by removing an individual ascomata and placing it into drop of sterile water. The ascospores released into the water are spread onto PDA for growth.

Concluding remarks

Species concepts in *Colletotrichum* have evolved from the use of basic morphological

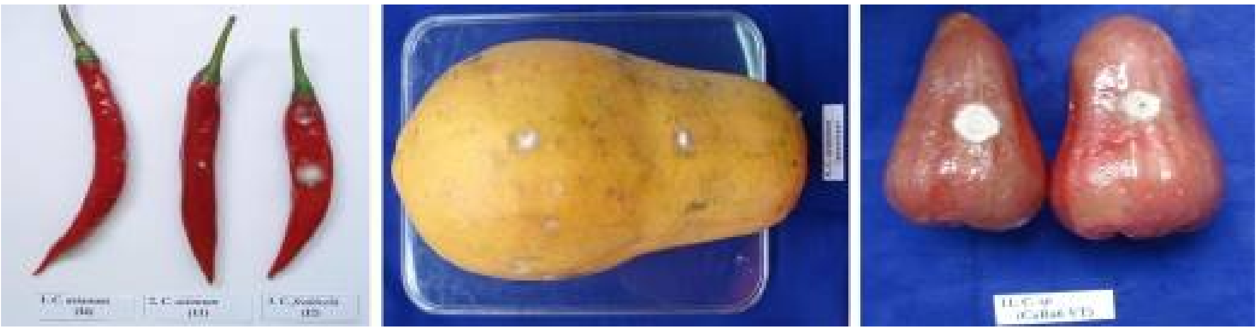

Fig. 6. Symptom on fruits 7 days after inoculation.

Fig. 7. Symptom on leaves 7 days after inoculation.

data to a polyphasic scrutiny incorporating a powerful molecular component. This has resulted in traditional classification being less practical, as holotypes or other types are presently not very useful as DNA cannot usually be extracted.

It is recommended that a polyphasic approach should be adopted in future studies before new species of *Colletotrichum* are introduced or epitypes are designated, and should incorporate molecular, morphological, physiological, and pathogenic data. In the following section we summarise the recommended protocols.

Protocols for describing new species and epitypes

1. A detailed morphological description of the fungus should be provided (see *Colletotrichum fructicola* Prihastuti, L. Cai & K.D. Hyde, Fungal Diversity 39: 96 and *C. anthrisci* Damm, P.F. Cannon & Crous, Fungal Diversity 39: 12 for examples).
2. In addition to any distinctive morphological or other polyphasic characters, the proposed new species should show sufficient evolutionary divergence from other closely related taxa based on a multiple gene sequence analysis, and it is essential that any comparisons should be made with type specimens.
3. It is recommended that multiple gene loci should be characterized such as ITS, TUB2, GPDH, ACT and others. Sequences should be deposited in a recognised international database.
4. Media used for descriptions and comparison should be as recommended in this paper. Where possible a description should also be provided from a collection on host tissues.
5. The ex-type cultures of any new *Colletotrichum* species should be deposited in at least two internationally recognised culture collections (preferably more), and all information should be registered in MycoBank.
6. Details of growth rate in a standard medium at a standard temperature and growth condition should be included.
7. Three basic rules should be taken into account when considering the suitability of a specific specimen and derived culture to serve as epitype and ex-epitype (Mcneill *et al.*, 2006). The strain should be A) from the original host, B) from the original geographic locality and C) have well-

matched morphology and other phenotypic characters as the type which should be examined if available and preferentially illustrated. When an epitype is designated, the holotype, lectotype, or neotype that the epitype supports must be explicitly cited (Mcneill *et al.*, 2006).
8. Pathogenicity testing may be useful and should be carried out where possible.

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