Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand

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Thirty-four isolates of *Colletotrichum* spp. were isolated from banana, ginger, *Euphatorium thymifolia*, soybean, longan, mango and *Draceana sanderiana*. They included endophytes from healthy plants and probable pathogens from disease lesions. Isolates were identified and grouped based on colony morphology, and size and shape of appressoria and conidia. Molecular analysis based on sequences of the rDNA internal transcribed spacers (ITS1 and ITS2), indicated that the *Colletotrichum* isolates comprised four clades that paralleled the morphological groupings. Most isolates clustered within three distinct clades which potentially represented different species. Endophytes isolated from different hosts are more likely to be the same species. *Colletotrichum musae* was positioned close to the *C. gloeosporioides* clades. Morphological and phylogenetic analysis of *Colletotrichum* pathogens and endophytes showed that endophytic isolates were most similar to *C. gloeosporioides* however, no pathogenic isolates clustered with endophytic isolates. The correlation between morphological and molecular-based clustering demonstrated the genetic relationships among the isolates and species of *Colletotrichum* and indicated that ITS rDNA sequence data were potentially useful in taxonomic species determination.

Key words: *Colletotrichum*, endophytes, ITS, molecular analysis, pathogens, taxonomy.

Introduction

The genus *Colletotrichum* Corda contains many morphologically similar taxa comprising endophytic, saprobic and plant pathogenic fungi (Kumar *et al*., 2004; Photita *et al*., 2004). *Colletotrichum* species cause anthracnose, which

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can cause considerable damage in a large number of crops such as cereals, coffee and legumes (Bailey and Jeger, 1992; Lenné, 1992). Even greater economic losses are due to post harvest anthracnose disease of tropical and subtropical fruit such as avocado, banana and mango (Mordue, 1967; Jeffries *et al*., 1990). *Colletotrichum* species are also found on decaying wild fruits (Tang *et al*., 2003).

Colletotrichum species that cause serious plant disease are also commonly isolated as endophytes from healthy plants, and have been identified as saprobes on dead plant material (Photita *et al*., 2001a, 2003, 2004; Promputtha *et al*., 2002; Toofanee and Dulymamode, 2002; Kumar and Hyde, 2004). Endophytic, saprobic and many pathogenic strains in the genus have been frequently classified as *Colletotrichum gloeosporioides* or *Colletotrichum* sp. (Brown *et al*., 1998; Bussaban *et al*., 2001; Photita *et al*., 2001b, 2003; Promputtha *et al*., 2002). *Colletotrichum gloeosporioides* is a commonly isolated endophyte from a range of plant species (Rodrigues, 1994; Brown *et al*., 1998; Bussaban *et al*., 2001; Photita *et al*., 2001b). Strains of *C. gloeosporioides* and *Colletotrichum musae* were also detected as latent pathogens in banana (Jeger *et al*., 1995). Therefore, it is important to establish the relationship among strains of various *Colletotrichum* isolates with different life forms and to establish diversity of the species.

Differentiation between *Colletotrichum* species based on host range or host origin may not be reliable, since taxa such as *C. acutatum*, *C. gloeosporioides*, *C. graminicola* and others infect a broad range of host plants (Sutton, 1980, 1992). Traditional identification and characterization of *Colletotrichum* species has relied primarily on differences in morphological features such as colony colour, size and shape of conidia and appressoria, optimal temperature for growth, growth rate, presence or absence of setae, and existence of the *Glomerella* teleomorph (von Arx, 1957; Smith and Black, 1990; Gunnell and Gubler, 1992; Sutton, 1992). Due to environmental influences on the stability of morphological traits and the existence of intermediate forms, these criteria are not always adequate for reliable differentiation among *Colletotrichum* species. The overlap of morphological characters and phenotypes among species makes classification difficult. Molecular techniques provide alternative methods for taxonomic studies and are important tools in solving the problems of species delimitation (MacLean *et al*., 1993).

Arbitrarily primed polymerase chain reaction (AP-PCR) and polymorphisms in nuclear DNA, ribosomal DNA (rDNA), mitochondrial DNA (mtDNA), and AT-rich DNA have been utilized to differentiate among populations of *C. acutatum*, *C. coccodes*, *C. fragariae*, C. *gloeosporioides*, *C.*

kahawae, *C. magna* and *C. orbiculare* (Correll *et al*., 1993; Freeman *et al*., 1993; Hodson *et al*., 1993; Sreenivasaprasad *et al*., 1993; Sherriff *et al*., 1994; Brown *et al*., 1996; Johnston and Jones, 1997). Due to lower conservation of nucleotide sequences in the nontranscribed and internal transcribed spacer (ITS) regions between the small and large nuclear rDNA subunits than in the coding regions, these have been used to detect recent evolutionary divergence within *Colletotrichum* species (Freeman *et al*., 2000; Ford *et al*., 2004). Also, species specific primers have been designed primarily according to dissimilarities in the sequence of the ITS regions of representative isolates of *Colletotrichum* from different species (Mills *et al*., 1992; Hodson *et al*., 1993; Sreenivasaprasad *et al*., 1993; Brown *et al*., 1996; Johnston and Jones, 1997; Ford *et al*., 2004).

The objectives of this study were to investigate the diversity of the genus *Colletotrichum* isolated from tropical plants in Thailand to (1) establish if classification of *Colletotrichum* species using morphological characters is supported by molecular sequence analysis, (2) establish if pathogenic and endophytic *Colletotrichum* strains on the same host are the same species, and (3) establish if morphologically similar endophytic *Colletotrichum* strains on different hosts were the same species.

Materials and methods

Isolation of plant pathogenic fungi

Colletotrichum isolates were obtained from lesions on leaf, petiole and fruit of banana (*Musa acuminata*), longan (*Lunguas galanga*), mango (*Mangifera indica*), an ornamental plant (*Draceana sanderiana*) and soybean (*Glycine max*) from Chiang Mai, Thailand. Five *ca*. 5 × 5 mm, pieces of tissue were taken from healthy, and the margin of diseased tissue, surface sterilized and then placed on the surface of Potato Dextrose Agar (PDA). Samples were sterilized by dipping in 10% sodium hypochlorite for 3-5 minutes and washing in 2 or 3 series of sterile water. Plates were incubated at room temperature (28-30°C) and observed periodically. The growing edges of any fungal hyphae developing from the leaf disks were then transferred aseptically to corn meal agar slants. The fungi were identified following sporulation and pure cultures were stored at 4°C on PDA slants.

Isolation of endophytic fungi

Endophytes were isolated from leaves, petioles and pseudostems of healthy plants of banana (*Musa acuminata*), ginger (*Alpinia malaccensis*) and

Euphatorium thymifolia from Doi Suthep Pui National Park, Chiang Mai, Thailand. Plant tissues were surface sterilized to remove epiphytic fungi. The leaves were first washed in running water. All leaf discs and segments were surface sterilized in 75% ethanol for 1 min, 1% sodium hypochlorite for 3 minutes and 95% ethanol for 0.5 min and then dried on sterilized paper. Five surface sterilized leaf discs and segments were evenly spaced in Petri dishes (9 cm diam) containing 2% (w/v) malt extract agar (MEA) supplemented with Rose Bengal (30 mg/l) to slow fungal growth, and streptomycin sulfate (50 mg/l) to suppress bacterial growth. The mycelium developed within two weeks and the fungi were then transferred to corn meal agar slants. The fungi were identified following sporulation and pure cultures were stored at 4 °C on PDA slants.

Morphological examination

Malt extract agar (MEA) and Richard's V8 medium (RV8) were inoculated with mycelial discs (5 mm diam.) taken from the growing edge of colonies. Cultures were grown in darkness at 20, 25 and 30°C. Colony diam. was recorded daily (three replicates, two measurements per replicate) for 1 week. Growth rate was calculated as the 7-day average of mean daily growth (mm per day). Size and shape of conidia were recorded from the colonies grown on PDA plates at room temperature (28-30°C). Conidia were taken from actively growing colonies mounted in lactic acid, and examined for size and shape. Data were analyzed using analysis of variance ($P<0.05$) and Duncan's multiple range test with SPSS software version 10.0 (SPSS Inc., Chicago, USA).

Colony morphology was noted after 7 days. Appressoria were produced using slide culture techniques, where 10 mm squares of sterile PDA were placed in an empty Petri dish, the edge of the agar was inoculated with spores taken from a sporulating culture, and a cover slip was placed over the inoculated agar (Johnston and Jones, 1997). Appressoria formed across the underside of the cover slip and their shape and size were then recorded.

DNA extraction

Each culture was derived from a single conidium from the original isolate and was subsequently maintained in Potato Dextrose Broth. Cultures were incubated without shaking at room temperature for 3-5 days. Mycelia were harvested by vacuum filtration onto a Watman No.1 filter paper disk.

After rinsing with sterile water, approximately 0.5g of mycelium for each sample was frozen in liquid nitrogen and ground in a sterile mortar. Nucleic acid was extracted using the adapted CTAB method of Ford *et al*. (2000). The total genomic DNA concentration was estimated by absorbance at 260 nm.

Polymerase chain reaction

The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S rDNA, were amplified by PCR using Primers ITS4 and ITS5 (White *et al*., 1990). The PCR reaction was carried out using a PTC-200 thermocycler (MT Research Inc., USA). Amplification was performed in 25 µl volumes with the same conditions as described by Phan *et al*. (2002). PCR products were separated by electrophoresis in 1.4% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator. Each amplification reaction was replicated three times.

Cloning and sequencing

PCR products were purified from agarose using the Concert Rapid Gel Extraction System (Gibco BRL, Life Technologies, Australia). Purified fragments were then ligated into the pGEM-T Easy vector (Promega, USA) and transformed into *Escherichia coli* JM109 (Promega, USA) using heat shock at 42°C for 3 min. Five colonies were randomly selected and their insert sizes assessed using PCR with the M13 primer sites. Selected colonies were grown in LB-Ampicillin (100 µg/ml) liquid medium overnight at 37°C in the dark with rotary shaking. Plasmid DNA was extracted using the alkaline lysis method (Sambrook *et al*., 1989). The DNA was sequenced using the ABI PRISM BigDye Terminater Cycle Sequencing Reaction Kit (Perkin Elmer-Applied Biosystems, USA) and analyzed on an ABI 377 automated DNA sequencer.

Sequence analysis

Sequences were deposited in GenBank and compared against those sequences already found in the databases using the Basic BLAST search option of BLAST 2.0 (http://www.ncbi.nlm.nih.gov/BLAST). Sequence data for related fungi were obtained from GenBank as follows: AF272788 *Glomerella acutata*; AF272780 *G. cingulata*; AJ536227 *C. coccodes*; AB042303 *C. higginsianum*; AB042313 *C. boninense*; AB087221 *C. fragariae*; AJ536228 *C.*

kahawae; AB087220 *C. musae* and AF451899 *C. truncatum*. ITS1 and ITS2 including 5.8S sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al*., 1994). Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002). Parsimony trees were obtained using the heuristic search options with 1000 replications of random stepwise sequence additions and tree-bisection-reconnection (TBR) branch swapping for the exact solution. The tree stability was evaluated by 1000 parsimony bootstrap replicates (Felsenstein, 1985). Phylogenetic trees were constructed from distance matrix values by the neighbor-joining (NJ) method (Saitou and Nei, 1987). NJ trees were constructed using distances produced with the Jukes-Cantor (one-parameter) method (Jukes and Cantor, 1969) or the K2P (two-parameter) method (Kimura, 1980). A bootstrap analysis was performed using 1000 resamples of the data. *Neurospora bonaerensis* (AJ002029) was used as an outgroup.

Results

Morphological comparisons

Twenty-four *C. gloeosporioides* isolates were obtained as endophytes from healthy plants of banana, ginger, and *E. thymifolia*. Twelve pathogenic isolates of *Colletotrichum* spp. were obtained from typical anthracnose lesions found on leaves of banana – *C. musae*; mango, longan, *D. sanderiana* – *C. gloeosporioides*; and soybean – *C. truncatum* (Table 1).

Distinct morphological culture types were recognized (Table 2, Fig. 1). There were three distinct groups within *C. gloeosporioides* (Table 2). Group 1 comprised 15 isolates; 12 endophytes (5 from banana, 4 from *E. thymifolia*, 3 from ginger) and 3 pathogens (1 each from mango, longan and *D. sanderiana*). The cultures had sparse, cottony, white to pale grey mycelium with abundant mycelia containing bright orange conidial masses produced in concentric rings on the colonies (Fig. 1a). Setae on the conidiomata were abundant. Group 2 comprised all endophytes – three isolates from banana and two from *E. thymifolia*. The cultures contained dense, white mycelium with a few orange conidial masses near the inoculum point (Fig. 1b) and conidiomata lacked setae. Group 3 contained endophytes from banana (3) and ginger (4). Colonies contained sparse, pale grey to black mycelium with a few orange conidial masses near the inoculum point (Fig. 1c), sclerotia were abundant and setae were present. The *C. musae* isolates were pathogenic on banana. The cultures were distinct with fast growing sparse aerial mycelium, white, with copious cinnamon conidial masses, conidia usually elliptical and setae absent (Fig. 1d).

Table 1. Isolates representing each of the morphological groups of *Colletotrichum* from Chiang Mai, Thailand used in this study and their host.

Isolate no.	Host	Life mode	GenBank Acc.#
C. musae			
CMUBP-1	*Musa acuminata*	Pathogen	AY266401
PDC 093	*Musa acuminata*	Pathogen	AY266397
PDC147	*Musa acuminata*	Pathogen	AY266399
C. gloeosporioides group 1			
CMUBE 1814	*Musa acuminata*	Endophyte	AY266378
CMUBE 1850	*Musa acuminata*	Endophyte	AY266395
CMUBE 1840	*Musa acuminata*	Endophyte	AY266388
CMUBE 1851	*Musa acuminata*	Endophyte	AY266391
CMUBE 1852	*Musa acuminata*	Endophyte	AY266392
G 2	*Euphatorium thymifolia*	Endophyte	AY266405
G133	*Euphatorium thymifolia*	Endophyte	AY266402
G 136	*Euphatorium thymifolia*	Endophyte	AY266382
G1849	*Euphatorium thymifolia*	Endophyte	AY266383
CMUZE 0028	*Alpinia malaccensis*	Endophyte	AY266385
CMUZE 0439	*Alpinia malaccensis*	Endophyte	AY266389
CMUZE 0040	*Alpinia malaccensis*	Endophyte	AY266381
PDC 001	*Draceana sanderiana*	Pathogen	AY266380
PDC 002	*Mangifera indica*	Pathogen	AY266390
PDC 113	*Lunguas galanga*	Pathogen	AY266394
C. gloeosporioides group 2			
CMUBE 1098	*Musa acuminata*	Endophyte	AY266374
CMUBE 1099	*Musa acuminata*	Endophyte	AY266376
CMUBE 1820	*Musa acuminata*	Endophyte	AY266377
G 102	*Euphatorium thymifolia*	Endophyte	AY266393
G 140	*Euphatorium thymifolia*	Endophyte	AY266375
C. gloeosporioides group 3			
CMUBE 812	*Musa acuminata*	Endophyte	AY266400
CMUBE 1535	*Musa acuminata*	Endophyte	AY266403
CMUBE 1815	*Musa acuminata*	Endophyte	AY266404
CMUZE 0015	*Alpinia malaccensis*	Endophyte	AY266398
CMUZE 0036	*Alpinia malaccensis*	Endophyte	AY266387
CMUZE 0357	*Alpinia malaccensis*	Endophyte	AY266369
CMUZE 0041	*Alpinia malaccensis*	Endophyte	AY266379
C. truncatum			
PDC 003	*Glycine max*	Pathogen	AY266371
PDC 005	*Glycine max*	Pathogen	AY266386
PDC 006	*Glycine max*	Pathogen	AY266384

Table 2. Conidial size, shape and growth rate at room temperature on PDA for the morphological groups of *Colleotrichum* spp.

	Conidial shape	Length (μm)			Width (μm)			Growth rate on PDA (mm d^{-1})		
		Min	Max	Mean*	Min	Max	Mean*	20°	25°	30°
C. gloeosporioides group 1	Cylindrical	12	20	16.1^{c}	3	6	4.6^{d}	8.6^{b}	11.4^{b}	11.6^{b}
C. gloeosporioides group 2	Cylindrical	12	16	14.3^{d}	6	8	6.1^{b}	7.4^{c}	9.3^{c}	9.9^{c}
C. gloeosporioides group 3	Cylindrical	12	24	20.9^{b}	6	10	8.4^{a}	6.5^{d}	8.4^{d}	8.2^{d}
C. musae	Elliptical or cylindrical	12	20	14.1^{d}	4	10	5.7^{c}	11.6^{a}	12.6^{a}	12.5^{a}
C. truncatum	Falcate	20	31	23.1^{a}	3	4	3.8^{e}	5.7^{e}	7.1^{e}	7.4^{e}

*The mean difference is significant at the 0.05 level.

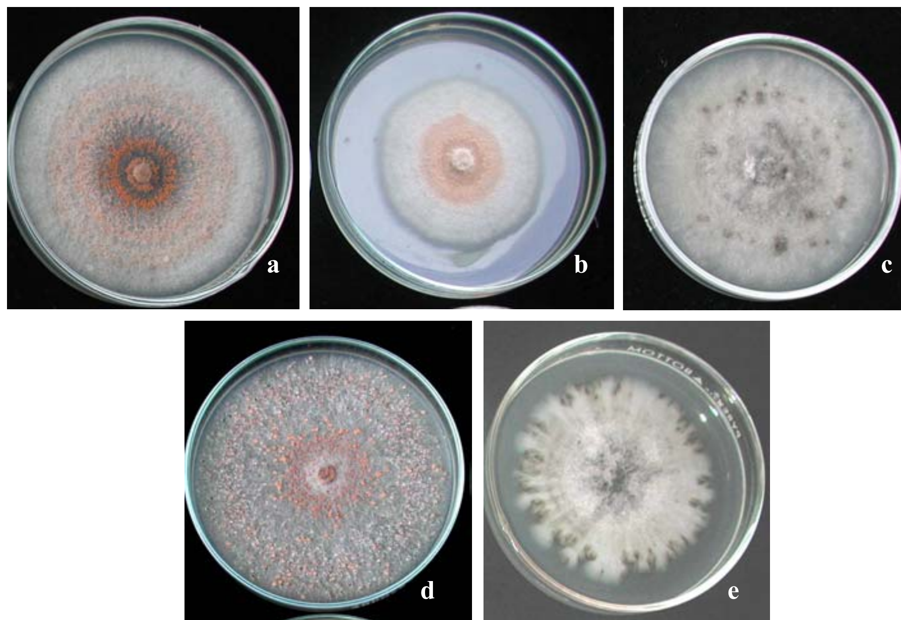

Fig. 1. Colonies of *Colletotrichum* species on PDA; **a.** *C. gloeosporioides* group 1; **b.** *C. gloeosporioides* group 2; **c.** *C. gloeosporioides* group 3; **d.** *C. musae*; **e.** *C. truncatum*.

The *C. truncatum* isolates were pathogenic on soybean. The cultures had dense aerial mycelium, were dark brown to black or greyish felt mycelium. Tufting of mycelium was irregular, with honey-coloured conidial masses, conidia were falcate and setae on conidiomata were abundant (Fig. 1e).

Spore shape was similar for each of the *C. gloeosporioides* isolates (Table 2). However, spore size was significantly different for each *C. gloeosporioides* group with those of group 3, being the largest (Table 2). All isolates produced both mycelial appressoria and appressoria directly from germ tubes. Appressoria of *C. gloeosporioides* were clavate, while those of *C. musae* and *C. truncatum* were more irregular than *C. gloeosporioides*. Differences in growth rates of the various *Colletotrichum* isolates studied were most apparent at 25ºC (Table 2) and there was no difference in growth rate between isolates grown on MA and RV8 media.

Sequence analysis of the ribosomal DNA spacer sequence (ITS)

The total size of the ITS1 and ITS2 regions, including the 5.8S rDNA gene, of the isolates studied varied from 581 to 620 bp. Maximum parsimony analysis of the rDNA sequences gave 98 parsimonious trees and strict consensus of these is shown in Fig. 2. One thousand maximum parsimony bootstrap replicates were performed and high bootstrap replication percentages were given on the tree's internal nodes (Fig. 2). The topologies of the neighbor-joining trees, constructed using both the Kimura 2 parameter and Jukes-Canter distance matrices, were very similar, and were consistent with the maximum parsimony consensus tree (results not show).

Phylogenetic analysis grouped the *Colletotrichum* isolates into four clades (Fig. 2). All *Colletotrichum* isolates that clustered in clade I included four reference isolates from GenBank; AB087220 (*C. musae*), AB087221 (*C. fragariae*), AF272780 (*G. cingulata*) and AB536228 (*C. kahawae*) with 97% bootstrap support. At the top of this clade was the reference isolate (*C. musae*) and three sequences of banana pathogens and one sequence of an endophyte from ginger, while *C. kahawae* sequence was placed at the basal end of the clade. Four *C. truncatum* soybean pathogens grouped together in Clade II along with the reference isolate AF451899 (*C. truncatum*) with high bootstrap support (100%). At the top of Clade III was one endophyte isolate from *E. thymifolia* grouped with *G. acutata* with 100% bootstrap support and the other branch of this clade comprised one endophyte from *E. thymifolia* and two endophytes from banana with 100% bootstrap support. The basal end of the clade included three sequences of endophytes from banana and the reference sequence from *C. boninense*, with 100% bootstrap support. Clade IV included

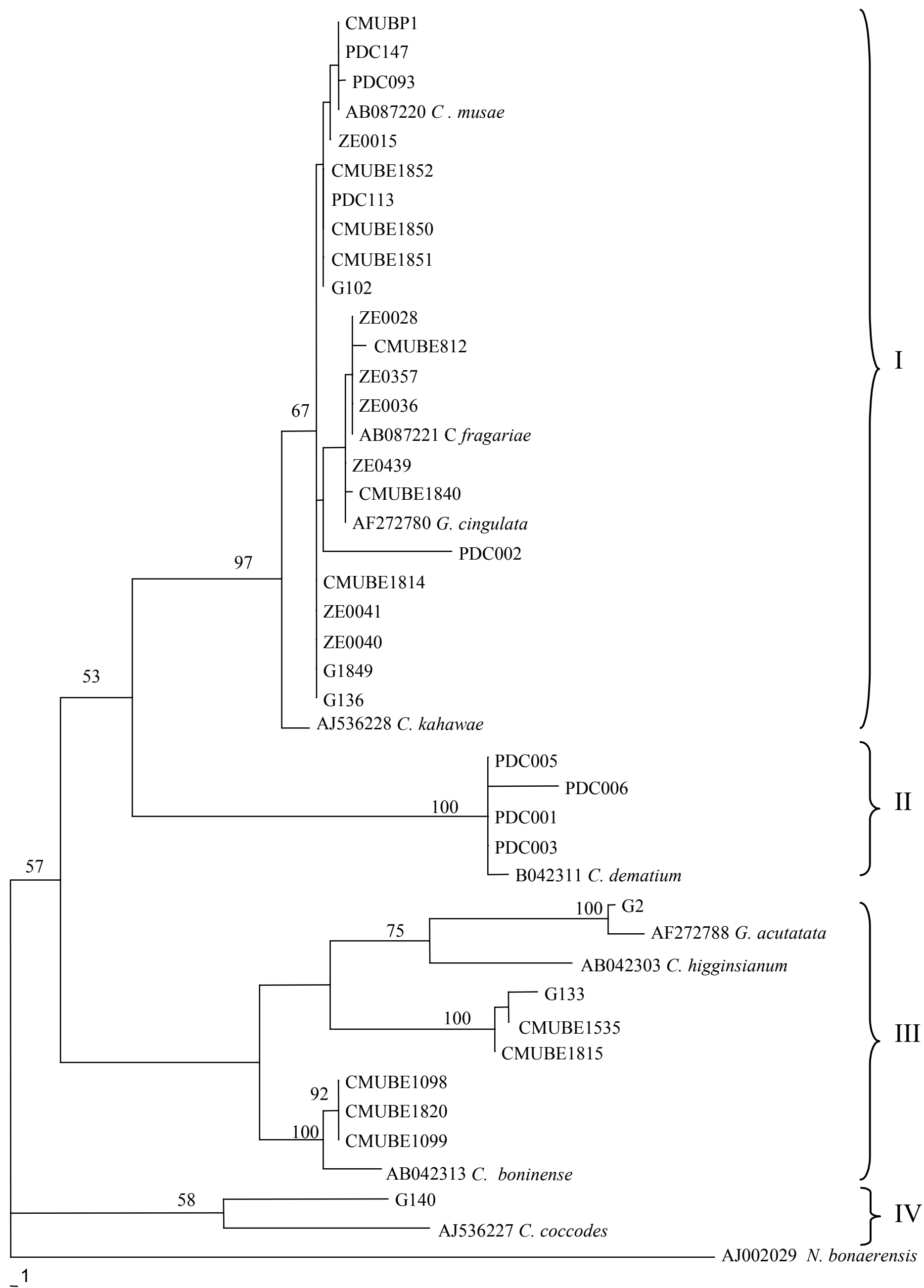

Fig. 2. Parsimonious tree obtained by heuristic search in a phylogenetic analysis of ITS1, ITS2 including rDNA sequences data from *Colletotrichum* from various hosts, as well as reference isolates. Bootstrap confidence levels, based on 1000 resamples are given on the appropriate branches.

one endophytic isolate from *E. thymifolia* and one reference sequence from Genbank AJ536227 (*C. coccodes*).

Discussion

Morphological and cultural characters were used to group 36 *Colletotrichum* isolates into species *C. musae*; *C. gloeosporioides* groups I, II and III; and *C. truncatum*. Sequence comparison of ITS regions of these isolates from banana, ginger and *E. thymifolia* against published sequences confirmed them as *Colletotrichum* species. Sequence data also correlated with the morphological grouping of these species.

The pathogenic isolates from banana were morphologically similar to the description of the banana pathogen *C. musae* (Sutton, 1980; Price, 1995) and ITS sequences correlated with the reference taxon *C. musae*. An endophyte isolate from ginger also clustered within this group but did not have similar morphological characteristics to the *C. musae* isolates. The ginger isolate needs to be further tested for pathogenicity on banana to confirm its taxonomy and life form. *Colletotrichum musae* has generally been recognized as a distinct species from *C. gloeosporioides* (Holliday, 1980; Sutton, 1980; Jones, 2000). RAPD banding patterns of isolates of *Colletotrichum* isolated from banana in Australia also indicated that the species were distinct from other *Colletotrichum* species (Mills *et al*., 1992).

The grouping of *C. gloeosporioides* into three subgroups indicated the complexity of this species. There appeared to be at least three distinct species of *Colletotrichum* identified based on morphological and molecular characteristics. *Colletotrichum gloeosporioides* group 1 comprised the largest group with isolates from several hosts and included a GenBank sequence of *Glomerella cingulata*. Basal to the main cluster was *C. kahawae*. Sreenivasaprasad *et al*. (1993) used restriction fragment length polymorphism analyses of ribosomal and mitochondrial DNA; and sequence analyses, and found a high degree of molecular similarity between *C. gloeosporioides*, *C. fragariae* and *C. kahawae*, suggesting that these should not be considered as separate species (Freeman *et al*., 1993; Buddie *et al*., 1999; Martínez-Culebras *et al*., 2002). *Colletotrichum kahawae* causes coffee berry disease and is morphologically similar to *C. gloeosporioides* but has been distinguished from *C. gloeosporioides* by its ability to infect green berries and by biochemical characteristics (Waller *et al*., 1993). Nucleotide sequences of the internally transcribed spacer 1 region of the rDNA repeat unit of *C. kahawae* and *C. gloeosporioides* isolates from *Coffea* spp. differed by only two to three bases (98.8-98.2% homology) confirming the close genetic relationship of *C.*

kahawae to *C. gloeosporioides*. However, caution should be exercised when relying on ribosomal ITS sequence data to discriminate related taxa due to the limited number of informative sites identified. Other regions of the genome, such as the β-tubulin gene, have been identified as suitable for phylogenetic reconstruction (Crous *et al*., 1999).

Colletotrichum acutatum (anamorph of *Glomerella acutata*) infects a wide range of hosts, which includes strawberry (Wilson *et al*., 1990; Harris, 1992). *Colletotrichum acutatum* can occur on strawberry plants as cryptic infections and sometimes in lesions in complexes with *C. gloeosporioides* and *C. fragariae*. Previous research has broadened the taxonomic definition of *C. acutatum*, largely on the basis of molecular data (Sreenivasaprasad *et al*., 1994; Reed *et al*., 1996) and threatens the practical utility of this taxonomic name.

Colletotrichum gloeosporioides group 2 comprised three endophytic isolates from banana and one endophytic isolate from *E. thymifolia*. These isolates were identified as *Colletotrichum* based on morphological characters as *Colletotrichum* and were more similar to *C. gloeosporioides* than to other species. *Colletotrichum boninense* also clustered with this group. However, these isolates may not be *C. gloeosporioides* even though there was relatively high sequence similarity with GenBank sequence AF272780 *G. cingulata* (*C. gloeosporioides* group 1). The nucleotide divergence of rDNA is variable in different fungal taxa, and there are no definite criteria for the rDNA nucleotide divergence for inter-specific and intra-specific levels at present. Moriwaki *et al*. (2003) showed that the ITS1 region of *C. boninense* was 190 bp, whereas for *C. gloeosporioides* this region was 171 bp. Consequently, the difference between *C. boninense* and *C. gloeosporioides* should reflect interspecific relationships.

Colletotrichum gloeosporioides group 3 included two endophytic isolates from banana and one endophytic isolate from *E. thymifolia*. The phenotypic and molecular data indicated that this was a separate and possibly new species. The morphological characteristics differed from all other *C. gloeosporioides* groups in that the spores were generally larger and the colonies different. The isolates in this group did not correspond to any of the *Colletotrichum* species described by Sutton (1980) and was most similar to *C. gloeosporioides*.

There were four pathogenic isolates of *C. truncatum* isolated from soybean, which clustered with a GenBank sequence for *C. truncatum* (AF451899). These isolates had falcate conidia and clustered at the base of the parsimony tree. They did not cluster with any other isolates from other hosts, indicating that this pathogen may be host-specific to soybean. However, Ford *et al*., (2004) recently showed 99.8% identity among rDNA sequences of *C. truncatum* that was isolated from soybean (*Gycine max*), common bean

(*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*). This indicated a near complete conservation in ITS sequence between isolates from different host species.

Morphological and phylogenetic analysis of *Colletotrichum* pathogens and endophytes showed that endophytic isolates were most similar to *C. gloeosporioides*. No pathogenic isolates clustered with endophytic isolates. The ITS sequences demonstrated that from the parsimony tree the pathogenic isolates from banana were different from the endophytic isolates.

Colletotrichum endophytes isolated from banana, ginger and *E. thymifolia* clustered into the three morphological subgroups of *C. gloeosporioides*. Endophytes isolated from different hosts are, therefore, more likely to be the same species. This may have important implications for biodiversity estimates. If this small subset of *Colletotrichum* endophytes from different hosts are the same species, then fungal endophyte species numbers may be lower then suspected (Hawksworth, 2001). However, since three species are also apparent in this small subset of *Colletotrichum gloeosporioides* one could predict that species numbers may be higher than expected.

In summary *Colletotrichum* species were differentiated based on sequence analysis and the results were comparable to differentiation of taxa based on morphological and cultural characteristics. Lacap *et al*. (2003) have shown that some morphotypes concur well with species when tested using molecular data. In this study most *Colletotrichum* isolates from the same host with the same morphological characteristics were shown using molecular data to be the same species. *Colletotrichum* isolates from the same host but with different morphological characteristics were considered to be different species and this was supported by molecular data. The only exception was the isolate CMUBE812, an endophyte from banana, which had similar morphological characteristics but clustered in a separate group in the phylogenetic analysis. If more taxa from various hosts and regions were sequenced and analyzed it is likely to reveal many more species of *Colletotrichum*.

Molecular studies are helping to resolve relationships within the genus *Colletotrichum*, but studies to date can be regarded only as preliminary. Many questions remain regarding the evolutionary relationships within the genus and a consensus needs to be confirmed using taxonomic structure. When more *Colletotrichum* isolates from different hosts are surveyed and sequenced, a better understanding of the genus will be obtained. Sequence analysis from ITS regions has made some progress towards a better understanding of the taxonomy of *Colletotrichum*, but further molecular analysis using other genes and more sensitive approaches are required (Cunnington *et al*., 2004).

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