MOLECULAR DIVERSITY OF *COLLETOTRICHUM* ISOLATES FROM TROPICAL FOREST TREE SPECIES AND THEIR RELATIONSHIP WITH OTHER KNOWN *COLLETOTRICHUM* SPECIES

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MAZIAH, Z., BAILEY, J. & NASH, C. 2003. Molecular diversity of *Colletotrichum* isolates from tropical forest tree species and their relationship with other known *Colletotrichum* species. The present study reveals species grouping in the genus *Colletotrichum* isolated from the tropical forest trees. The sequence data of domain 2 of the ribosomal DNA provided further evidence to distinguish isolates of *Colletotrichum*, which could not be achieved by morphological and cultural criteria studies alone. Analysis of the 13 forest tree isolates suggests that they represent three main groups. Group 1 consists of only one isolate (689). Group 2 consists of isolates 634, 635, 640, 645, 657, 659, 664 and 665. Of these isolates 645, 657, 659 and 665 were identical. Group 3 consists of isolates 630, 660, 662 and 674. The maximum percentage nucleotide difference between the groups was small, merely 5.2% (94.8 % similarity). Comparison of the forest isolates with other *Colletotrichum* species revealed that the forest isolates corresponded to some of the known species. Thus, some of the forest isolates had sequences that were identical to the defined species. In Group A, isolate 640 was identical to a *C. gloeosporioides* from *Digitalis* (120), whereas isolates 645, 657, 659 and 665 were identical to *C. gloeosporioides* from *Aeschynomene* (074) and *Mangifera* (501). Isolate 689 was identical to *C. acutatum* from *Musa* (058) and *Lupinus* (163). In Group B, isolate 660 was identical to *G. cingulata* from *Phaseolus* (529 and 531) and *Vigna* (238). The morphological comparison of the isolates in each group is also presented.

Key words: Morphology - forest tree isolates - DNA sequencing

MAZIAH, Z., BAILEY, J. & NASH, C. 2003. Kepelbagaian molekular isolat *Colletotrichum* yang diperoleh daripada spesies pokok hutan dan hubung kaitnya dengan spesies *Colletotrichum* yang telah dikenal pasti. Kajian ini menunjukkan terdapat beberapa kumpulan daripada genus *Colletotrichum* yang diperoleh daripada pokok hutan. Data jujukan domain 2 rDNA memberikan penjelasan yang mendalam bagi membezakan isolat-isolat *Colletotrichum* tersebut. Perbezaan ini tidak dapat

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dilihat dengan hanya menggunakan kaedah morfologi dan kultur. Analisis 13 isolat yang diguna menunjukkan isolat-isolat tersebut mewakili tiga kumpulan utama. Kumpulan 1 cuma mengandungi satu isolat sahaja iaitu 689. Kumpulan 2 mengandungi isolat 634, 635, 640, 645, 657, 659, 664 dan 665. Walau bagaimanapun isolat 645, 657, 659 dan 665 adalah serupa. Kumpulan 3 mengandungi isolat-isolat 630, 660, 662 and 674. Peratusan maksimum perbezaan nukleotid antara kumpulan-kumpulan tersebut adalah sangat kecil, cuma 5.2% (94.8% persamaan). Perbandingan isolat *Colletotrichum* daripada pokok hutan dengan spesies *Colletotrichum* yang lain menunjukkan isolat daripada pokok hutan menyerupai beberapa spesies yang telah dikenal pasti. Oleh itu terdapat beberapa isolat daripada pokok hutan yang jujukannya menyamai spesies tertentu. Di dalam kumpulan A, isolat 640 menyerupai *C. gloeosporioides* daripada *Digitalis* (120) manakala isolat 645, 657, 659 dan 665 menyerupai *C. gloeosporioides* daripada *Aeschynomene* (074) dan *Mangifera* (501). Daripada kumpulan B isolat 660 menyerupai *G. cingulata* daripada *Phaseolus* (529 dan 531) dan *Vigna* (238). Perbandingan morfologi bagi isolat setiap kumpulan juga diberikan.

Introduction

Identification of *Colletotrichum* species based on morphological and cultural criteria has many limitations as species, such as *C. gloeosporioides,* are extremely variable. A species may be identified as one or the other species depending on the criteria that are accorded most significant by the individual researcher. Moreover, morphology alone does not allow identification of strains at the physiological level. When breeding programmes, control initiatives and quarantine procedures require this type of accuracy, diagnosis based on morphology is of limited value (Sutton 1992).

Generally, the most significant advances in taxonomy are coming from approaches based on analysis of nucleic acids. These include analysis of restriction fragment-length polymorphisms (RFLPs), polymerase chain reaction (PCR), analysis of random amplified polymorphic DNAs (RAPDs) and DNA sequencing (Williams *et al.* 1990, Kohn 1992).

RFLP analysis was used to determine the genetic relationship within *C. gloeosporioides* isolates which cause anthracnose on *Stylosanthes* species (Braithwaite *et al.* 1990). Similar methods were used to differentiate various isolates of *C. gloeosporioides* from worldwide collections (Hodson *et al.* 1992, Mills *et al.* 1992). RAPD analysis was used to assess the molecular variation of 40 *C. gloeosporioides* isolates from different hosts (Mills *et al.* 1992). The coffee berry disease pathogen was recognised as a distinct group within *C. gloeosporioides* using the same method (Sreenivasaprasad *et al.* 1993). Molecular marker, based on RFLPs and RAPD, was used to genetically group *C. gloeosporioides* from tropical fruits (Alahakoon *et al.* 1994).

DNA sequencing is another method to analyse nucleic acids. Many analyses of DNA sequences have been based on rDNA because it occurs in high copy numbers, possesses conserved as well as variable sequences, and can be amplified and sequenced with universal primers based on their conserved sequences (Bruns

et al. 1991, Stackebrandt *et al.* 1992). The use of large ribosomal subunit (LRSU) is increasing because different regions of the LRSU, i.e. domains 1 and 2 and the internal transcribed spacer (ITS 1 and 2), show more variation in their evolutionary rate than the domains of small ribosomal subunit. They are, therefore, useful in distinguishing more closely related taxa (Kohn 1992).

Analysis of the variable ITS regions of rDNA was used to clarify the molecular variation of isolates of *C. gloeosporioides* and other *Colletotrichum* species which infect fruit crops (Mills *et al.* 1992, Sreenivasaprasad *et al.* 1993). Sreenivasaprasad *et al.* (1994) used the ITS 1 region of rDNA to identify various isolates of *Colletotrichum* and found them to be *C. acutatum.* Likewise, sequence comparison of ITS 2 and domains 1 and 2 of rDNA from various isolates of *Colletotrichum* were used by Sherriff *et al.* (1994). Their results suggest that rDNA sequence data of these individual regions can be used to distinguish between species of *Colletotrichum.* The other *Colletotrichum* species used for comparison were *C. gloeosporioides* from *Mangifera, C. gloeosporioides* from *Aeschynomene, C. gloeosporioides* from *Digitalis, C. acutatum* from *Musa, C. acutatum* from *Lupinus, G. magna* from *Cucumis, G. cingulata* from *Vigna, G. cingulata* from *Phaseolus, C. lindemuthianum* from *Phaseolus, C. malvarum* from *Sida, C. orbiculare* from *Cucumis* and *C. orbiculare* from *Xanthium.* They were chosen as representatives of several distinct morphological groups, but with emphasis on those with straight conidia that attack different hosts. Their sequence data were derived from previous studies by Sherriff *et al.* (1994).

The main aim of this study was to assess the genetic diversity among the isolates of *Colletotrichum* from forest trees through analysis of their rDNA sequences and to reveal their taxonomic relationships with other known species of *Colletotrichum.*

Materials and methods

Fungal isolation

In the present study *Colletotrichum* isolates were obtained from diseased materials collected at several forest nurseries. Tissues bearing disease symptoms were surface-sterilised in commercial clorox (5–6% sodium hypochlorite) for 4 min and rinsed in three changes of sterilised distilled water. After drying on sterilised filter paper the tissues were plated on Potato Dextrose Agar (PDA) and incubated at room temperature (27–30 °C) for two days. Each individual mycelium growing from the tissue pieces was transferred onto fresh PDA plate and cultivated at room temperature until sporulation occurred. The isolates were identified according to their morphological characteristics. They were numbered and stored under oil in the laboratory for future use. The details of the 13 isolates used in this study are described in Table 1. All the isolates were tentatively identified as *C. gloeosporioides.* Morphological studies on these isolates were conducted; details of these studies were dealt with in another paper (Maziah & Bailey 2000). Only results are discussed in this paper.

Table 1 The isolate numbers, hosts and symptoms of the *Colletotrichum* isolates used in this study

Isolate no.	Original host	Disease symptom	Collection site
630	*Acacia mangium*	Leaf spots and lesions	FRIM, Malaysia
634	*Hevea brasiliensis*	Leaf spots	Dengkil, Malaysia
635	*Chrysalidocarpus lutescens*	Leaf spots	FRIM, Malaysia
640	*Schizostachym branchycladium*	Leaf spots	FRIM, Malaysia
645	*Magnolia malayana*	Leaf lesions	FRIM, Malaysia
657	*Calamus manan A*	Leaf spots and lesions	FRIM, Malaysia
659	*C. manan B*	Leaf spots and lesions	FRIM, Malaysia
660	*Pterocarpus indicus A*	Leaf spots and lesions	FRIM, Malaysia
662	*P. indicus B*	Leaf spots and lesions	FRIM, Malaysia
664	*P. indicus* C	Leaf spots and lesions	FRIM, Malaysia
665	*P. indicus D*	Leaf spots and lesions	FRIM, Malaysia
674	*Schoutenia accrescens*	Leaf spots	FRIM, Malaysia
689	*Gliricida sepium*	Leaf spots	Gualan, Guatemala

FRIM = Forest Research Institute Malaysia

DNA extraction

For DNA extraction, the isolates were grown in V8 Czapek Dox liquid medium for four days at 25 °C. The mycelium was harvested by vacuum filtration through muslin cloth and immediately frozen in liquid nitrogen and ground into powder. DNA was extracted using modified protocol of Graham *et al.* (1994). A volume of 1 ml of hot extraction buffer (CTAB, Sigma; 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) was added to 1 g mycelial powder in a 1.5 ml microcentrifuge tube and mixed by gentle inversion. The tube was then incubated at 55 °C for 20 min. After incubation the tube was centrifuged for 10 min at 13 000 r.p.m. in a microcentrifuge. The supernatant was collected and one volume of chloroform was added and mixed by gentle inversion for 2 min before centrifugation at 13 000 r.p.m. for 30 s. The upper aqueous phase was collected, and placed in a 2 ml microcentrifuge tube. Following this, 0.1 volume of 4 M sodium acetate and 2 volumes of ice-cold absolute alcohol were added and mixed by gentle inversion. The samples were placed in a freezer at - 80 °C for 20 min to precipitate. The tube was then centrifuged at 13 000 r.p.m. for 1 min and the supernatant discarded. The DNA was washed twice with 70% ethanol, mixing gently by inversion. Finally the DNA pellet was dried using a heating block (37 °C), dissolved in 1X TE (1 ml), and stored at - 20 °C. DNA concentration was estimated using UV spectrophotometer (Diode Array, Hewlett Packard, USA) by absorbance at 260 nm.

PCR amplification

Sequence analysis was based on domain (D)2 of the LRSU ribosomal region. The PCR primers used were Pn 2_(5'GTTCACCATCTTTCGGGTCC3') and Pn 9_(5'CTTAAGCATATCAATAAGCGGA-GG3'). These primers were synthesised

by the University of Bristol. All reactions were performed in 10 µl 10 × PCR buffer, overlaid with mineral oil. The buffer included 100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 80 µM of each dNTP (Pharmacia), 2 µl of each primer, 10 µl genomic DNA, 2.5 units of Taq DNA polymerase (Sigma), and sterile distilled water to make the volume up to 100 µl. PCR reactions were carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, USA), using the following programme: initial denaturation at 96 °C for 2 min, then 30 cycles of 15 s at 96 °C (denaturing), 30 s at 60 °C (annealing), 60 s at 72 °C (elongation), followed by a final elongation of 3 min at 72 °C and then cooling at 4 °C. After amplification, the PCR product was analysed by electrophoresing 1 µl aliquot on 0.8% agarose stained with TBE/ethidium bromide, and image was visualised under UV light.

DNA sequencing and analysis

Sequencing reactions were carried out using the sequenase PCR product sequencing kit protocols (USB-Amersham International plc, USA). DNA was sequenced using Primer Pn 4_(5'CCTTGGTCCGTGTTTCAAGACGGG 3'). Sequencing reaction mixes were run in gels containing 8% acrylamide (NBL, Gene Science Ltd, UK), in a vertical electrophoresis system (Base Runner Nucleic Acid Sequencer, Int. Biotech. Inc. USA). Sequencing gels were read manually. The sequenced regions were aligned manually on the basis of alignments given for D2 region of *Colletotrichum* in Sherriff *et al.* (1994).

The complementary distance matrix of pair-wise sites with the same nucleotide was calculated from the sequence data, with all differences (transitions, transversions, insertions and deletions) given the same weight. The trees showing the relatedness of isolates were constructed from the complementary distance matrix by the neighbour-joining method using the MEGA 1993 Version 1.0 software package. A bootstrap analysis using 1000 resamples of the sequence data was also carried out.

Results

rDNA sequence analysis of Colletotrichum *from forest trees*

The aligned nucleotide sequences of the D2 region (178 bases), along with data of the other previously sequenced *Colletotrichum* species (Sherriff *et al.* 1994), are presented in Table 2. The maximum number of base differences along the aligned sites was 7, i.e. 3.93%, with 11 positions showing changes across the set of isolates. The distance matrix, which indicated the nucleotide differences in the 178 sites, showed that there were very small differences between the isolates, with a maximum of 5.2% (i.e. between isolates 635 and 660, 689 and 660, and 634 and 662). The listance matrix also revealed that isolates 645, 657, 659, and 665 were very similar.

Table 2 Aligned sequences of D2 region of rDNA of *Colletotrichum* isolates

Isolate	10	20	30	40	50	60
501	AAAAGGGAAG	CGCTTGTGAC	CAGACTTGCG	TCCGGTGAAT	CACCCAGCTC	TCGCGGCTGG
074	----------	----------	----------	----------	----------	----------
645	----------	----------	----------	----------	-----*----	----------
657	----------	----------	----------	----------	-----*----	----------
659	----------	----------	----------	----------	-----*----	----------
665	----------	----------	----------	----------	-----*----	----------
664	----------	----------	----------	----------	-----*----	----------
120	----------	----------	----------	----------	----------	----------
640	----------	----------	----------	----------	-----*----	----------
635	----------	----------	----------	----------	-----*----	----------
634	----------	----------	----------	----------	-----*----	----------
058	----------	----------	----------	----------	----------	----------
163	----------	----------	----------	----------	----------	----------
689	----------	----------	----------	----------	-----*----	----------
630	----------	----------	----------	----------	-----*----	----------
662	----------	----------	----------	C---------	-----*----	----------
687	----------	----------	----------	----------	-----*----	----------
674	----------	----------	----------	C---------	-----*----	----------
238	----------	----------	----------	C---------	----------	----------
531	----------	----------	----------	C---------	-----*----	----------
529	----------	----------	----------	C---------	-----*----	----------
660	----------	----------	----------	C---------	-----*----	----------
009	----------	----------	----------	C----C-G--	-----*G---	-------C--
076	----------	----------	----------	C----C-G--	-----*G---	-------C--
414	----------	----------	----------	C----C-G--	-----*G---	-------C--
083	----------	----------	----------	C----C-GG-	-----*G---	-------C--
465	----------	----------	----------	C----C-G--	---G**----	-------C--

Isolate	70	80	90	100	110	120
501	GGCACTTCGC	CGGCTCAGGC	CAGCATCAGC	TCGCTGTCGG	GGACAAAAGC	TTCAGGAACG
074	----------	----------	----------	----------	----------	----------
645	----------	----------	----------	----------	----------	----------
657	----------	----------	----------	----------	----------	----------
659	----------	----------	----------	----------	----------	----------
665	----------	----------	----------	----------	----------	----------
664	----------	----------	----------	----C-----	----------	----------
120	----------	----------	----------	----------	----------	----------
640	----------	----------	----------	----------	----------	----------
635	----------	----------	----------	----------	----------	----------
634	----------	----------	----------	----C-----	----------	----------
058	----------	----A-----	----------	-T--C-----	----------	----------
163	----------	----A-----	----------	-T--C-----	----------	----------
689	----------	----A-----	----------	-T--C-----	----------	----------
630	-------T--	----------	----------	-T--C-----	----------	---G------
662	-------T--	----------	----------	-T--C-----	----------	---G------
687	-------T--	----------	----------	----C-----	----------	---G------
674	----------	----------	----------	----C-----	----------	---G------
238	-------T--	----------	----------	----C-----	----------	---G------
531	-------T--	----------	----------	----C-----	----------	---G------
529	-------T--	----------	----------	----C-----	----------	---G------
660	-------T--	----------	----------	----C-----	----------	---G------
009	------C---	----------	----------	----------	----------	---G------
076	------C---	----------	----------	----------	----------	---G------
414	------C---	----------	----------	----------	----------	---G------
083	------C---	----------	----------	----------	----------	---G------
465	------C---	----------	----------	----------	----------	---G------

continued

Table 2 (continued)

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                130        140        150        160        170
501  TAGCTCTCT* TCGGGGAGTG TTATAGCCTG TTGCATAATA CCTTCGGCGG GCTGAGGT
074  ---------- ---------- ---------- ---------- ---------- --------
645  ---------- ---------- ---------- ---------- ---------- --------
657  ---------- ---------- ---------- ---------- ---------- --------
659  ---------- ---------- ---------- ---------- ---------- --------
665  ---------- ---------- ---------- ---------- ---------- --------
664  ---------- ---------- ---------- ---------- ---------- --------
120  ---------- ---------- ---------- -----C---- ---------- --------
640  ---------- ---------- ---------- -----C---- ---------- --------
635  ---------- ---------- ---------- ---------- A----A---- --------
634  ---------- ---------- ---------- ---------- A----A---- --------
058  -G----*--C ---------- ---------- ---------- ---------- --------
163  -G----*--C ---------- ---------- ---------- ---------- --------
689  -G----*--C ---------- ---------- ---------- ---------- --------
630  ---------- ---------- --------C- -----C---- ---------- --------
662  ---------- ---------- --------C- -----C---- ---------- --------
687  ---------- ---------- --------C- -----C---- ---------- --------
674  ---------- ---------- --------C- C----C---- ---------- --------
238  ---------C *--------- --------C- C----C---- ---------- --------
531  ---------- ---------- --------C- C----C---- ---------- --------
529  ---------- ---------- --------C- C----C---- ---------- --------
660  ---------- ---------- --------C- C----C---- ---------- --------
009  -G----*--C *--------- --------C- -----C---- -------T-- --------
076  -G----*--C *--------- --------C- -----C---- -------T-- --------
414  -G----*--C *--------- --------C- -----C---- -------T-- --------
083  -G----*--C *--------- --------C- -----C---- -------T-- --------
465  -G----*--C *--------- --------C- -----C---- -------T-- --------
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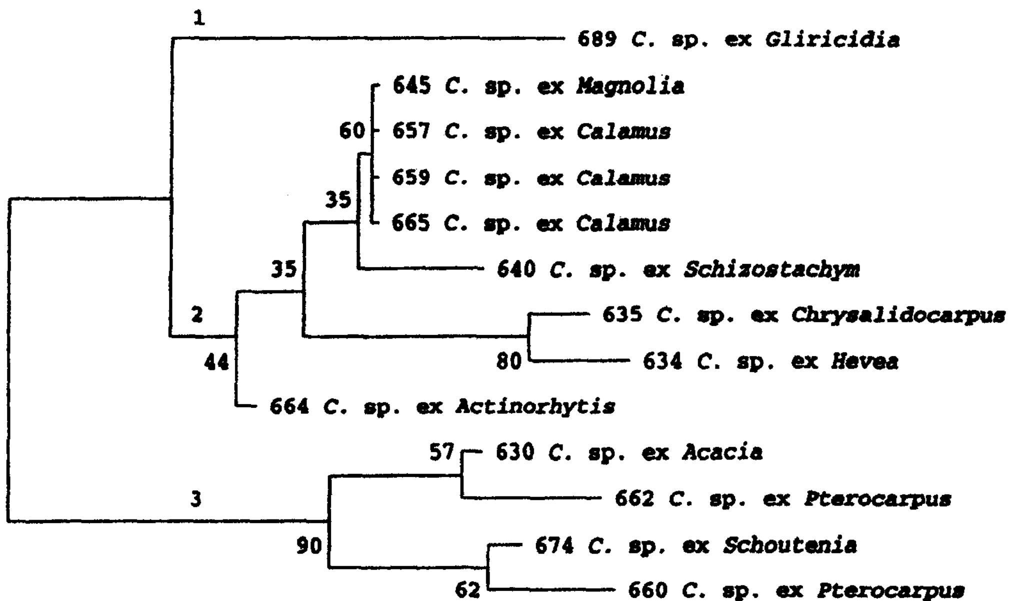
Key:
- = Identical to 501; * = Deletion

501 = *C. gloeosporioides* ex *Mangifera*
074 = *C. gloeosporioides* ex *Aeschynomene*
645 = *C.* sp. ex *Magnolia*
657 = *C.* sp. ex *Calamus*
659 = *C.* sp. ex *Calamus*
665 = *C.* sp. ex *Calamus*
664 = *C.* sp. ex *Actinorhytis*
120 = *C. gloeosporioides* ex *Digitalis*
640 = *C.* sp. ex *Schizostachym*
635 = *C.* sp. ex *Chrysalidocarpus*
634 = *C.* sp. ex *Hevea*
058 = *C. acutatum* ex *Musa*
163 = *C. acutatum* ex *Lupinus*
689 = *C.* sp. ex *Gliricidia*
414 = *C. orbiculare* ex *Cucumis*
083 = *C. lindemuthianum* ex *Phaseolus*
465 = *C. orbiculare* ex *Xanthium*
662 = *C.* sp. ex *Pterocarpus*
076 = *C. malvarum* ex *Sida*
009 = *C. lindemuthianum* ex *Phaseolus*
660 = *C.* sp. ex *Pterocarpus*
529 = *G. cingulata* ex *Phaseolus*
531 = *G. cingulata* ex *Phaseolus*
238 = *G. cingulata* ex *Vigna*
674 = *C.* sp. ex *Schoutenia*
687 = *G. magna* ex *Cucumis*
630 = *C.* sp. ex *Acacia*

The neighbour-joining tree, which illustrates the relationship of the isolates, revealed that the isolates from forest nurseries could be divided into three main groups (Figure 1). The first group (1) consisted of a single isolate (689). The second group (2) contained most of the isolates (8 isolates). Its first branch was divided into two; one branch ended with isolate 664 and the other was further divided into several levels of subbranches, consisting of isolates 634, 635, 640, 645, 657, 659 and 665. Isolates 645, 657, 659 and 665 were identical and they were closely related to isolate 640. On the whole, within group 2, the percentage



Scale: each — is approximately equal to the distance of 0.0529%

Figure 1 Neighbour-joining tree illustrating the relationship between isolates of *Colletotrichum* from forest trees based on DNA sequence

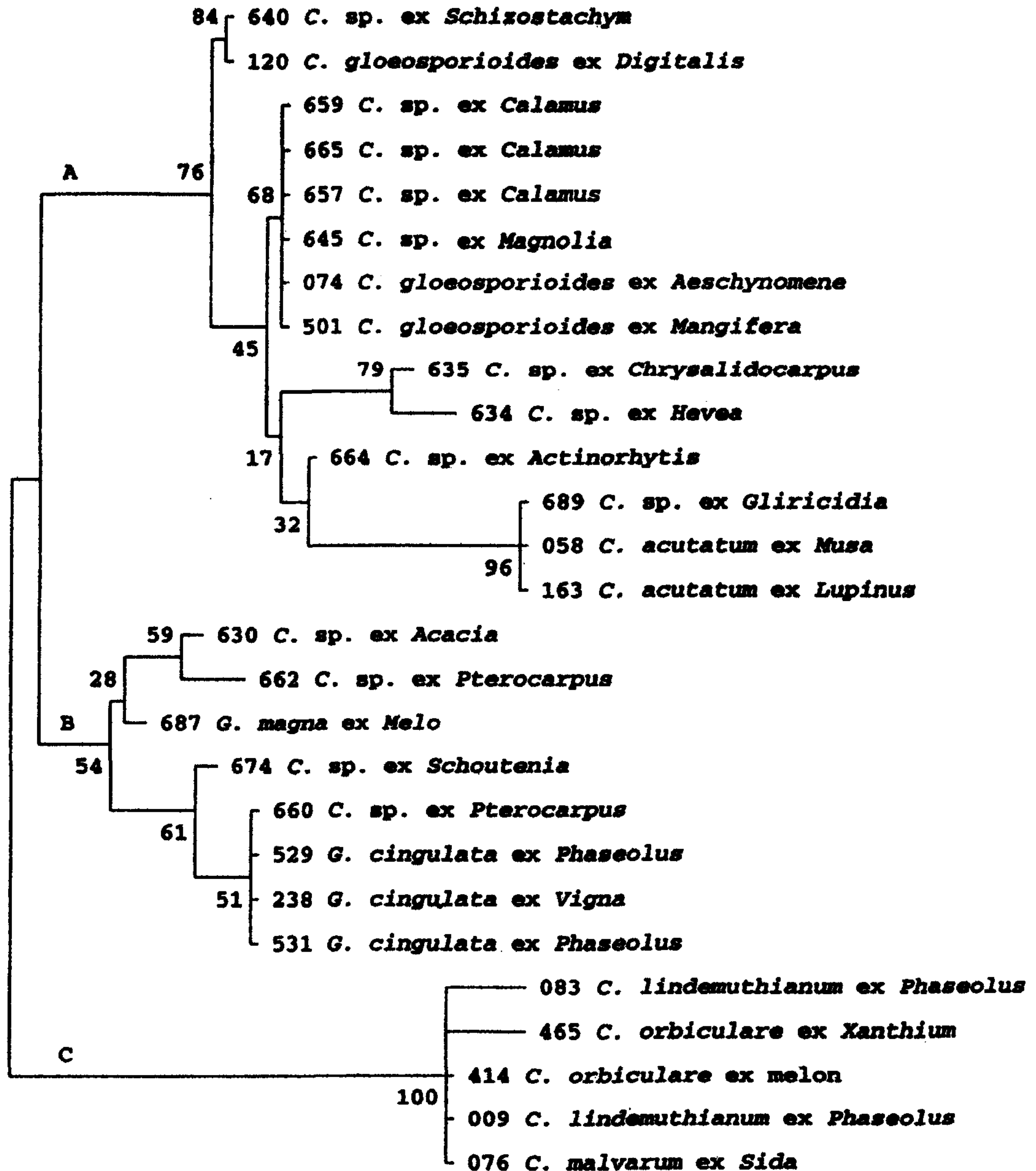
nucleotide difference ranged from 0.6 to 2.3%, with isolates 635 and 640 being the most diverse pair. The bootstrap values within group 2 varied from 35 to 80%, whilst the value between group 1 and 2 was 44%, indicating that isolate 689 was not consistently different to the isolates in group 2.

Group 3 consisted of only four isolates. The main branch was divided into two subbranches, and each of these subbranches contained a pair of isolates. The first pair contained isolates 630 and 662, while the second contained isolates 674 and 660. Within group 3 the percentage nucleotide difference ranged from 0.6 to 2.3%, with isolates 630 and 674 being the most diverse pair. The bootstrap value showed that the first level of branch of this group was very reliable. Between groups, the values for the range of percentage nucleotide difference were group 1/2: 1.7 to 3.5%, group 1/3: 3.5 to 5.2% and group 2/3: 2.9 to 5.2%.

rDNA sequence analysis of Colletotrichum *species from forest trees as well as other host species*

The maximum number of base differences along the aligned 178 sites was 16 or 8.99% (Table 2). Across the set of isolates, 24 position changes occurred; of these, 10 changes occurred in the last 58 sites. The distance matrix suggests that the percentage difference between the whole set of isolates ranged from 0.6 to 8.1%, with isolates 634 and 083, and 634 and 465 being the most diverse pairs.

The neighbour-joining tree revealed three main groups (Figure 2). The first group (A) consisted of 14 isolates. Nine isolates (634, 635, 640, 645, 657, 659, 664, 665 and 689) were from forest trees. Three isolates designated as *C. gloeosporioides*



Scale: each — is approximately equal to the distance of 0.1154%
Note: Bootstrap values (based on 1000 resamples) are given on appropriate branches

Figure 2 Neighbour-joining tree illustrating the relationship between isolates of *Colletotrichum* from forest trees and other host species based on DNA sequence

were isolates from *Digitalis* (120), *Aeschynomene* (074) and *Mangifera* (120). Another two isolates designated as *C. acutatum* were from *Musa* (058) and *Lupinus* (163). The second group (B) consisted of 8 isolates. Four (630, 662, 674 and 660) were forest tree isolates and three (529, 238 and 531) were isolates designated as *Glomerella cingulata* from beans, while the other was an isolate (687) designated as *G. magna* from melon. The final group (C), which was very distinct from groups A and B, consisted of isolates designated as *C. lindemuthianum* (083, 009), *C. malvarum* (076) and *C. orbiculare* (465, 414).

In group A, the percentage difference ranged from 0.6 to 3.5%. The sequence of isolates 657, 659, 645 and 665 from forest trees were similar to *C. gloeosporioides* from *Aeschynomene* (074) and *Mangifera* (501). Isolate 640 was similar to *C. gloeosporioides* from *Digitalis* (120). The sequence of isolate 689 from *Gliricidia* was identical to those of *C. acutatum* from *Musa* (058) and *Lupinus* (163). Although isolate 689 appeared to be more related to the *C. gloeosporioides* isolates (group A) than to the other species in groups B and C, the bootstrap value for this isolate was 96% indicating that it is distinct from the other members of group A. On this basis, isolate 689 is identified as a form of *C. acutatum.*

In group B, isolate 660 from *Pterocarpus* was identical to three other isolates of *G. cingulata* from *Phaseolus* (529 and 531) and *Vigna* (238). The rest of the isolates in this group differed slightly. Within this group the percentage difference ranged from 0.6 to 2.3%.

In group C, isolates 009, 076 and 414 were identical, while 083 and 465 were very closely related to the rest of the group. Within this group (C) the percentage difference ranged from 0.6 to 1.2%. Between groups, the values for the range of percentage difference were A/B: 2.3 to 5.2%, A/C: 5.2 to 8.1%, and B/C: 4.6 to 6.4%. Bootstrap analysis, as indicated in Figure 2, showed that group C was always distinct from groups A and B, but the distinction between groups A and B was not fully sustained.

Discussion

Ribosomal DNA (rDNA) sequence has been widely used to discriminate fungal taxa at the family, generic and subgeneric levels (Hillis & Dixon 1991, Lee & Taylor 1992, Cooke & Duncan 1997). Use of the large ribosomal subunit is increasing because different regions, i.e. the internal transcribed spacers 1 and 2, and domains 1 and 2 show more variation in their base compositions than do the domains of the small ribosomal subunit, which are more widely used for bacteria (Hillis & Dixon 1991, Kohn 1992).

Results of the present rDNA analysis revealed that there were three different groups within the 13 forest isolates. Group 1 consisted of an isolate (689) obtained from *Gliricidia sepium* in Guatemala (a non-Malaysian isolate). In group 2, four out of the eight isolates were identical, these included three isolates from *Calamus manan* and one from *Magnolia malayana.* The final group 3, consisted of four isolates of which two were from *Pterocarpus,* one from *Schoutenia accresscens* and one from *Acacia mangium.* This clearly shows that the same fungus can attack different hosts.

When the forest tree isolates were compared with other known *Colletotrichum* species, they corresponded to two of the three main branches of the neighbour-joining tree. In the first group, group A, isolates 640, 645, 657, 659 and 665 were very similar to isolates designated as *C. gloeosporioides*, including the 'Collego' strain (074) which is used as a mycoherbicide (Templeton 1992). All these isolates appeared to be typical of *C. gloeosporioides*. It is interesting to note, however, that isolate 689 was identical to isolates which were identified as *C. acutatum* (isolates 058 and 163) by comparison with data from Sreenivasaprasad *et al.* (1994), although the isolates had originally been designated as *C. gloeosporioides* on the basis of their host origin (Sherriff *et al.* 1994). Comparison of the isolates of *C. acutatum* and *C. gloeosporioides*, revealed a percentage difference ranging from only 1.7 to 3.5%. This was in contrast with data from Sreenivasaprasad *et al.* (1994), which showed that the divergence between *C. gloeosporioides* and *C. acutatum* based on the ITS 1 region was 15 to 17%. One possible reason for this, is that the D2 region does not have enough variable sites to distinguish the two species. Therefore, as suggested by Sreenivasaprasad *et al.* (1994), the ITS 1 region will probably be a more suitable site for distinguishing *C. acutatum* from other species.

The present study revealed that isolates characteristic of *C. gloeosporioides* or its teliomorph *G. cingulata*, and several forest tree isolates exist in different regions of the neighbour-joining tree (groups A and B). This was in agreement with Sherriff *et al.* (1994) who suggested that isolates presently termed *C. gloeosporioides* represent more than one species. Furthermore, alternative approaches based on RFLP and PCR-RAPD analysis also revealed considerable variation within *C. gloeosporioides*, but it has not been possible to relate this to the taxonomic status of this species (Sutton 1992).

Morphologically, all the forest isolates in this study, except 689, had ovoid conidia and were typical of *C. gloeosporioides* (Table 3). Furthermore, their conidia became septate during germination, a feature that eliminated them from being members of the *C. orbiculare* group, which does not produce septa during germination (Sherriff *et al.* 1994). This is consistent with the neighbour-joining tree, as none of the forest tree isolates were found associated with the *C. orbiculare* group (C). It was interesting to find isolate 689, which was morphologically distinct from the rest of the forest isolates, to be identical to those isolates which were designated as *C. acutatum*. Conidia of this isolate had acute apices, which are typical of *C. acutatum*. This is in discordance with the report by Boa and Lenné (1993) who identified 689 as *C. gloeosporioides*. Another morphological character which distinguished 689 from the rest of the forest isolates was its slow growth rate (Table 3). These characters have been used by several authors to distinguish *C. acutatum* from other *Colletotrichum* (Gunnel & Gubler 1992, Sutton 1992).

Three forest tree isolates (660, 662 and 674) in group B were notable for the production of very lobed appressoria as well as having broad conidia (Table 3). Isolates with conidial widths between 4.5 and 5.5 mm and obviously lobed appressoria should be classified as *C. crassipes* (Cox & Irwin 1988). Another morphological feature of these isolates was their abundant setae, which is also compatible with current definitions of *C. crassipes* (Sutton 1992). Therefore, on

Table 3 Morphological characteristics of *Colletotrichum* isolates from forest trees

Isolate		Conidial shape and apex	Mean conidial length (μm)	Mean conidial width (μm)	Appressorial shape	Septation	Perithecia	Setae	Growth rate (μm)
689 (A/1)	*C. acutatum*	Ovoid, acute	13.5 ± 2.18	4.6 ± 0.51	Globose, subglobose	Present	Absent	Absent	6.0 ± 0.50
634 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	17.9 ± 2.61	5.0 ± 0.40	Globose, subglobose	Present	Sterile	Present	13.6 ± 0.29
635 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	12.1 ± 4.48	5.0 ± 0.49	Globose, subglobose	Present	Absent	Present	4.3 ± 2.08
640 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	15.7 ± 2.49	5.0 ± 0.49	Subglobose, lobed	Present	Present	Present	12.1 ± 0.29
645 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	18.4 ± 2.42	5.5 ± 0.66	Globose, subglobose	Present	Absent	Absent	11.8 ± 2.52
657 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	16.9 ± 1.13	4.9 ± 0.35	Globose, subglobose	Present	Absent	Present	11.8 ± 1.53
659 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	12.7 ± 0.48	5.2 ± 0.66	Subglobose, lobed	Present	Absent	Absent	9.7 ± 1.44
664 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	17.5 ± 1.22	5.0 ± 0.55	Absent	Present	Sterile	Absent	11.1 ± 0.76
665 (A/2)	*C. gloeosporioides*	Ovoid, obtuse	18.4 ± 1.24	5.0 ± 0.30	Subglobose, lobed	Present	Sterile	Present	11.5 ± 1.32
630 (B/3)	*C. crassipes*	Ovoid, obtuse	12.8 ± 0.67	5.0 ± 0.34	Lobed	Present	Absent	Present	10.9 ± 0.58
660 (B/3)	*C. crassipes*	Ovoid, obtuse	17.7 ± 1.78	5.2 ± 0.55	Lobed	Present	Present	Present	14.2 ± 0.29
662 (B/3)	*C. crassipes*	Ovoid, obtuse	22.2 ± 3.61	7.8 ± 0.55	Lobed	Present	Absent	Present	9.2 ± 0.50
674 (B/3)	*C. crassipes*	Ovoid, obtuse	39.5 ± 2.65	10.7 ± 0.66	Lobed	Present	Absent	Present	8.9 ± 1.76

N.B. The isolate of 630 used for sequencing produced lobed appressoria

the basis of this, it is proposed that isolates within group B should be regarded as biological variants of a single species and they could be termed *C. crassipes.*

Isolate 660, besides having identical sequence, was also morphologically similar to three *G. cingulata* isolates (238, 529 and 531), as it produced fertile perithecia. However, isolate 640, which also produced fertile perithecia, was not in this group but in group A. On this basis, it can be concluded that the presence of perithecia cannot be used to distinguish species of *Colletotrichum.* This contradicts reports by several authors (Smith & Black 1990, Denoyes & Baudry 1995) who used the presence of perithecia to distinguish *C. gloeosporioides* from *C. fragariae* which cause anthracnose on strawberry.

In conclusion, this study has shown that the results of rDNA sequence data obtained with the forest trees isolates are fully supported by associated morphological data. This provides clear evidence that molecular analyses can be used to distinguish between species of *Colletotrichum.*

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