**In Press at Mycologia, published on May 27, 2010 as doi:10.3852/09-244**

Short title: *C. gloeosporioides* endophytes and pathogens

*Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panamá: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes

Enith I. Rojas

Stephen A. Rehner[1]

Gary J. Samuels

*USDA-ARS, Systematic Mycology and Microbiology Laboratory, Beltsville, Maryland 20705*

Sunshine A. Van Bael

Edward A. Herre

*Smithsonian Tropical Research Institute, Apartado 0843-03092, Republic of Panamá*

Paul Cannon

*CABI, Bakeham Lane, Egham, TW20 9TY, UK and Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK*

Rui Chen

Junfeng Pang

Ruiwu Wang

Yaping Zhang

*State Key Laboratories, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, Yunnan, People's Republic of China*

Yan-Qiong Peng

*Key Laboratory of Tropical Forest Ecology, Xinshiabanna Tropical Botanical Garden*

**Copyright 2010 by The Mycological Society of America.**

*Chinese Academy of Sciences, Kunming, People's Republic of China*

Tao Sha

*Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming 650091, People's Republic of China*

***Abstract:*** *Colletotrichum* interacts with numerous plant species overtly as symptomatic pathogens and cryptically as asymptomatic endophytes. It is not known whether these contrasting ecological modes are optional strategies expressed by individual *Colletotrichum* species or whether a species' ecology is explicitly pathogenic or endophytic. We explored this question by inferring relationships among 77 *C. gloeosporioides* s.l. strains isolated from asymptomatic leaves and from anthracnose lesions on leaves and fruits of *Theobroma cacao* (cacao) and other plants from Panamá. ITS and 5′-*tef1* were used to assess diversity and to delineate operational taxonomic units for multilocus phylogenetic analysis. The ITS and 5′-*tef1* screens concordantly resolved four strongly supported lineages, clades A–D: Clade A includes the ex type of *C. gloeosporioides*, clade B includes the ex type ITS sequence of *C. boninense*, and clades C and D are unidentified. The ITS yielded limited resolution and support within all clades, in particular the *C. gloeosporioides* clade (A), the focal lineage dealt with in this study. In contrast the 5′-*tef1* screen differentiated nine distinctive haplotype subgroups within the *C. gloeosporioides* clade that were concordant with phylogenetic terminals resolved in a five-locus nuclear phylogeny. Among these were two phylogenetic species associated with symptomatic infections specific to either cacao or mango and five phylogenetic species isolated principally as asymptomatic infections from cacao and other plant hosts. We formally describe two new species, *C. tropicale* and *C. ignotum*,

that are frequent asymptomatic associates of cacao and other Neotropical plant species, and epitypify *C. theobromicola*, which is associated with foliar and fruit anthracnose lesions of cacao. Asymptomatic *Colletotrichum* strains isolated from cacao plants grown in China included six distinct *C. gloeosporioides* clade taxa, only one of which is known to occur in the Neotropics.

***Key words:*** anthracnose, China, endophyte, multilocus, Panamá, pathogen

## INTRODUCTION

Species of *Colletotrichum* Corda are among the most commonly occurring pathogens and foliar endophytes of terrestrial plants and are recorded from approximately 2200 plant host species (Farr and Rossman 2009). As plant pathogens *Colletotrichum* spp. are the principal cause of anthracnose as well as pre- and postharvest fruit rots, damping-off and blossom and seedling blight diseases (Bailey and Jeger 1992). The genus is the subject of numerous studies that deal primarily with its role as a plant pathogen as summarized in Bailey and Jeger (1992) and Cannon et al. (2008). In addition to its conspicuous ecology as a plant pathogen *Colletotrichum* is also a ubiquitous asymptomatic foliar endophyte of a diverse spectrum of plant hosts (e.g. Lodge et al. 1996, Cannon and Simmons 2002, Gamboa and Bayman 2001, Lu et al. 2004, Duran et al. 2005, Morakotkarn et al. 2007, Osono 2008). The ecological significance of endophytism is unclear. Although it has been suggested that endophytic fungi might be quiescent saprobes (Petrini et al. 1995, Whalley 1996), latent pathogens (Stone et al. 2000) or mutualists (Herre et al. 2007, Mejía et al. 2008), specific examples detailing these hypotheses remain scant.

It has been shown that particular *Colletotrichum* endophytes confer protective benefits to cacao hosts by reducing disease incidence and damage caused by other plant

pathogens (Arnold et al. 2003, Herre et al. 2007). Mejía et al. (2008) reported the frequent isolation of *C. gloeosporioides* (Penz.) Penz. & Sacc. endophytes from cacao (*Theobroma cacao*) leaves. When conidial suspensions of an endophytic strain of the fungus were applied to cacao trees in flower or the early stages of fruit set, this treatment reduced pod loss from black pod disease (*Phytophthora palmivora*) in comparison to uninoculated controls (Mejía et al. 2008). Based on this evidence it was suggested that endophytic *Colletotrichum* could play a role in the biological control of cacao diseases. However *C. gloeosporioides* is a known pathogen of an exceptionally broad range of plant species, including cacao. The release of this fungus to control cacao diseases accordingly could pose an unacceptable hazard if endophytic strains also are capable of causing diseases in cacao or other plant species growing in the vicinity of cultivated cacao. The potential risk to non-target plant hosts could be mitigated however if it were demonstrated that asymptomatic *Colletotrichum* strains are both phylogenetically and ecologically distinct from pathogenic taxa.

Risk assessment of *C. gloeosporioides* as a potential biocontrol agent is difficult to conduct because the current morphological species concept (von Arx 1957) groups nonpathogenic and pathogenic strains together in this single species (Sutton 1980, 1992). Species circumscription and identification in *Colletotrichum* and its *Glomerella* teleomorph historically has been based on host range, characteristics on particular hosts and a suite of morphological characters, including conidial size and shape, presence or absence and morphology of setae, hyphal appressoria and growth characteristics on agar media. However available variation in these conventional taxonomic characters in *Colletotrichum*/*Glomerella* has failed to support the development of robust species

concepts because of character symplesiomorphy and homoplasy. In addition biological species concepts based on mating tests are impracticable due to the infrequent and unpredictable occurrence of the *Glomerella* teleomorph in both nature and in culture. Altogether there appears to be little prospect for devising comprehensive taxonomic identification procedures for *Colletotrichum* or its *Glomerella* teleomorph that rely solely on morphology, host associations or mating relationships (Crouch et al. 2009c).

Molecular phylogenetic approaches are increasingly used to characterize diversity in *Colletotrichum* (Johnston and Jones 1997, Mills et al. 1992, Moriwaki et al. 2003, Du et al. 2005), and the use of multilocus phylogenetics and phylogenetic species concepts (Taylor et al. 2000) are becoming an integral component of their systematic study (Johnston and Jones 2003; Guerber et al. 2003; Crouch et al. 2006; Farr et al. 2006; Liu et al. 2007; Crouch et al. 2009b, c; Damm et al. 2009; Prihastuti et al. 2009). However a basic phylogeny of *Colletotrichum* is lacking and there is as yet no consensus as to which genomic regions are most useful for differentiating isolates, delineating species boundaries or for resolving the backbone phylogeny of this cryptically diverse genus.

In this study we used single and multilocus nuclear sequence data to characterize asymptomatic and symptomatic *Colletotrichum* species associated with *Theobroma cacao* and other co-occurring indigenous Neotropical and non-indigenous plant species in Panamá. The objectives of this study were to (i) compare the effectiveness of two loci, the ITS and the intron-rich 5′ region of elongation factor-1α (5′-*tef1*), to detect and categorize genetic diversity among environmental isolates of *Colletotrichum*; (ii) resolve species boundaries and relationships within the *C. gloeosporioides* complex using multilocus phylogenetics and to contrast these results against OTUs provisionally

diagnosed with 5′-*tef1*; and (iii) evaluate the host ranges and ecological modes of these *Colletotrichum* species in light of the inferred phylogeny.

## MATERIALS AND METHODS

*Collection and cultures*.—*Colletotrichum* strains originated from multiple sources in Panamá and China 2004–2008 (SUPPLEMENTARY TABLE I): (i) endophytes of *Theobroma cacao* obtained from surveys conducted at five lowland mixed forest sites across the Isthmus of Panamá (Rojas et al. 2008); (ii) endophytes from seedlings or juvenile plants of six Neotropical tree species grown in a common garden plot in Gigante, Barro Colorado Monument, including the indigenous tree species *Anacardium excelsum*, *Genipa americana*, *Pentagonia macrophylla*, *Tetragastris panamensis*, *Trichilia tuberculata* and *Virola surinamensis*; (iii) endophytes from mature plants of *Cordia alliodora*, *Merremia umbellata* subsp. *umbellata* and *Zamia obliqua* from wet lowland forests; and (iv) anthracnose lesions of fruits and leaves of cultivated or feral cacao, mango (*Mangifera indica)*, avocado (*Persea americana)* and guanábana (*Annona muricata*). All Panamá host plant species are described in Croat (1978). In addition 28 *Colletotrichum* foliar endophyte strains were isolated from mature *T. cacao* leaves grown in Xishuangbanna Prefecture, Yunnan Province, China.

In the current work endophytes are defined as fungi isolated from asymptomatic leaves after rigorous surface sterilization (Arnold et al. 2003) and pathogens are defined as conidial or tissue isolates from anthracnose lesions on fruit and leaves. Single conidia were dispersed on potato dextrose agar (PDA; BBL, Sparks, Maryland) containing antibiotic (Rifampicin 0.5%). In cases where no conidia were observed on diseased hosts isolations were made directly from lesions with the same procedures used to isolate endophytes.

*Characterization of colony characters and morphology*.—Growth studies were performed with isolates subcultured on PDA and simple nutrient agar (SNA: Nirenberg 1976) at 25 C with a 12 h cool white fluorescent light/12 h dark photoperiod. A piece of sterile filter paper, ca. 20 $mm^2$, was placed on the surface of the solidified SNA to induce conidiation. In some cases conidiomata and conidia developed on sterilized pieces of carnation leaf placed on the surface of SNA.

Growth rates were determined from cultures at five temperatures (15, 20, 25, 30, and 37 C) in darkness. Inoculum for growth studies was taken from the margins of actively growing colonies grown on

cornmeal dextrose agar (Difco, Detroit, Michigan; cornmeal agar + 2% w/v dextrose) and placed in the middle of 20 mL test medium (PDA and SNA) in 100 × 15 mm Petri dishes. Measurements of colony diameter were taken at 24 h intervals over 5 d. Growth trials were replicated on three succeeding weeks, and the three series of measurements for each isolate were averaged.

Morphological observations were made at 3–10 d from cultures actively conidiating on PDA or SNA (with filter paper) under the photoperiod described above. Conidia, phialides and in some cases perithecia and ascospores were mounted in 3% KOH for microscopic observation. Hyphal appressorium development and morphology from representative strains for each resolved clade were observed on SNA slide cultures made by placing a block of SNA agar (ca. 7 × 7 mm) on a microscope slide, inoculating each of the four corners of the block with the test strain and placing an 18 × 18 mm cover slip over the block; slide cultures were incubated on a piece of moist filter paper in a Petri dish sealed with Parafilm™ and incubated in darkness 3 d.

Microscopic images were captured with the aid of a Nikon DXM1200 digital camera and Nikon ACT 1 software or with a Nikon Ds-Fi1 camera and NIS Elements Basic Research 2.30, SP4 (Nikon). Composite images were made for some structures with Helicon Focus 4.21.5 Pro (Helicon Soft, www.heliconfocus.com). Measurements were obtained with Scion Image (release Beta 4.0.2; Scioncorp, Frederick, Maryland). Data were analyzed statistically with Systat 10.0 (Wilkinson 2000).

Cultures were preserved on agar slants at 10 C, in 20% glycerol at −80 C, and as air-dried cultures on 5 × 20 mm filter paper strips at 10 C. Representative cultures and designated types and neotypes of *Colletotrichum* were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands (SUPPLEMENTARY TABLE I), and corresponding dried agar cultures were accessioned as voucher specimens in the U.S. National Fungus Collections (BPI).

*DNA extraction*, *PCR*, *sequencing*.—Mycelium for DNA extraction was grown at 125 rpm in potato dextrose broth (Difco) then washed in sterile distilled water and lyophilized. DNA was extracted following the manufacturer's protocol for fresh plant tissue with the PureGene genomic DNA isolation kit (Gentra Systems, Minneapolis, Minnesota).

Nucleotide sequences were determined for seven PCR-amplified nuclear markers including the nuclear ribosomal intergenic transcribed spacers 1 and 2 (ITS), two contiguous regions of elongation

factor-1α (5′-*tef1* and 3′-*tef1*), β-tubulin (*btub*), the 5′ end of RNA polymerase I largest subunit (*rbp1*), a partial sequence of DNA lyase (*apn2*), and the intergenic region between the 3′ ends of DNA lyase and the mating type gene MAT1-2 (*apn2/mat12igs*). (Information on primers used for PCR amplification and sequencing is in SUPPLEMENTARY TABLE II.)

PCR reactions were performed with a standard amplification profile starting with a denaturation cycle at 95 C for 2 min followed by 40 cycles of three steps that included a 30 s denaturation at 95 C, 30 s annealing at 50–56 C (temperatures optimized per locus or sample where necessary), an 80 s extension at 72 C, ending with a 72 C incubation 10 min. As needed primer annealing temperature adjustments or touchdown procedures (Don et al. 1991) were used to reduce or eliminate interfering, nonspecific PCR artifacts. PCR amplicons were gel-purified through 1.5% NuSieve (Cambrex, New Rutherford, New Jersey) preparative agarose gels. Gel-isolated amplicons were freeze thawed and extruded from the gel matrix by centrifugation and used as sequencing template. Bidirectional sequencing was performed with BigDye 3.1 (Applied Biosystems, Foster City, California) in 5 μL volumes at 1/16 the manufacturer's recommendation and the data collected on an ABI 3130*xl* automated sequencer.

*Data editing and phylogenetic analysis*.—Sequence data were assembled into contigs and edited with Sequencher 4.8 (GeneCodes Corp., Ann Arbor, Michigan). Multiple sequence alignments were created with the multiple sequence alignment program MAFFT (Katoh et al. 2005). The E-INS-i alignment strategy was used to align the 5′-*tef1*, whereas the G-INS-i option was used for all other loci. Manual adjustments to 5′-*tef1* alignments were required to correctly align exon-intron boundaries.

All seven gene partitions were analyzed individually by maximum parsimony (MP) and nonparametric MP bootstrapping (MP BS) analysis with PAUP* 4b10 (Swofford 2003; Sinauer Associates, Sunderland, Massachusetts). MP-based analyses were conducted with equal character weighting; where gaps were included (i.e. ITS and 5′-*tef1*) these were treated as missing data. MP searches for the shortest trees implemented tree-bisection and reconnection branch-swapping (TBR) and 1000 random-addition sequence replicates. MP BS searches to assess clade support employed 1000 pseudo replicates of the data, with 10 random-sequence additions per replicate, and TBR branch swapping.

In the first analysis MP and MP BS analyses of ITS and 5′-*tef1* were used to perform an initial screen of all 77 Panamanian isolates to delineate operational taxonomic units (OTUs) for morphological

and multilocus phylogenetic evaluation. Sequences from strains E2550, Q822 and Q855 were used to root both the ITS and 5′-*tef1* analyses.

In a second analysis a 29-taxon dataset with five gene partitions including 3′-*tef*, *btub*, *rbp1*, *apn2* and *apn2/mat12igs* was used to evaluate relationships among Panamanian *Colletotrichum* OTUs diagnosed in the 5′-*tef1* screen. Each data partition was analyzed under MP as described, and tree topologies from all data partitions were compared to determine whether any significant conflict existed among the trees. After concatenation the five-locus dataset was analyzed by MP as above and also under maximum likelihood, ML (ML BS) in GARLI 0951 (Zwickl 2006), and Bayesian inference, BI, with MrBayes 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). All multilocus analyses were rooted to clade C strains 3386, 4766 and 4801, which are positioned as the sister of the *C. gloeosporioides* clade in the ITS phylogenetic tree (FIG. 1b). The hierarchical ratio test (Posada and Crandall 1998) in MrModeltest 2.2 (Nylander 2004), in conjunction with PAUP*, was used to determine the best-fit model of nucleotide substitution for the individual and combined datasets for ML and BI analyses. Optimal models of sequence evolution selected by MrModeltest for the individual combined gene partitions used in the BI analysis were GTR + G for 3′-*tef1* and β-*tub*, HKY + G for *apn2* and *rpb1*, and HKY + I for the *apn2*/*Mat*1igs, and GTR + I + G for the combined dataset. ML nonparametric bootstrap analysis (ML BS) with GARLI was performed with 5000 generations without improving the topology parameter and 500 ML pseudoreplicates of the data. Bayesian posterior probabilities (BI PP) were determined with MrBayes 3.1 by performing four simultaneous Markov chains (three incrementally heated, one cold) of 1 000 000 generations, with a tree sampled every 100 generations, including the first. The log-likelihood values of all trees were inspected and the first 25% of trees were discarded to ensure that the remaining trees had converged on similar log-likelihood values. The remaining trees were imported into PAUP* and a 50% consensus tree computed with the support values for each branch constituting its posterior probability. The log-likelihood (−lnL) scores of the best tree from the GARLI ML search and the Bayesian consensus tree were calculated in PAUP*.

*Chinese* Colletotrichum *endophytes of cacao*.—MP analysis of 5′-*tef1* sequences was used to assess diversity among 28 Chinese *Colletotrichum* strains isolated as asymptomatic endophytes from cultivated cacao plants grown in China and their affinities to selected Neotropical taxa.

## RESULTS

*Diagnosing genetic diversity*.—MP and MP BS analyses of ITS and 5′-*tef1* sequences were performed as a proximate screening method to delimit ad hoc genetic groups within the *Colletotrichum* collection (FIG. 1a, b). The ITS and 5′-*tef1* alignments were submitted to TreeBASE (10327; www.TreeBASE.org).

Among *Colletotrichum* strains in this study, 5′-*tef1* haplotypes varied 322–520 bp. Predicted translations revealed that these differences stem from variation in the number and spatial configurations of introns (diagrammed in FIG. 2). A total of 10 distinct intron insertion sites were observed, although the number of introns present per individual haplotype was 2–4. Altogether there were 15 distinct 5′-*tef1* intron configuration types among the Neotropical isolates (FIG. 2). In addition strains within four intron configuration types (1, 4, 9, 10) showed varying levels of sequence divergence that partitioned these configuration types into two or more clades. Henceforth we refer to each distinct 5′-*tef1* terminal cluster as 5′-*tef1* subgroups and subgroups are defined as an exclusive terminal clade composed of individuals with identical intron configurations that also are identical or nearly so in sequence. The 5′-*tef1* subgroups henceforth are referred to with a three part alphanumeric convention according to (i) ITS clade, (ii) 5′-*tef1* terminal subclade and (iii) 5′-*tef1* configuration type (e.g, "A2-1": ITS clade A, 5′-*tef1* terminal clade 2, 5′-*tef1* intron configuration type 1). All 5′-*tef1* haplotype configuration types described here are listed adjacent to their corresponding intron configuration maps (FIG. 2). 5′-*tef1* introns 2.1–2.3 and 4.1–4.3 represent two clusters of three intron insertions sites located at three adjacent nucleotide positions (FIG. 2). Because of sequence similarity among introns at different insertion sites within each cluster it is

possible that the locations of introns have shifted due to the phenomenon of intron sliding (Rogozin et al. 2000). The tight spacing of introns within each of these clusters complicates their alignment, and manual adjustments to alignments are necessary to correctly align these insertion sites. However identical results in the diagnosis of terminal diversity were achieved regardless of whether the different introns in each cluster were aligned according to insertion site or whether introns from the three adjacent insertion sites within each cluster were forced into alignment with one another. Results reported here are based on introns aligned according to insertion sites.

Results of both the ITS and 5′-*tef1* analyses yielded concordant support for four deep lineages that are provisionally termed clades A–D (FIG. 1a). (Tree statistics for the ITS and 5′-*tef1* analyses are in TABLE I, and MP BS nodal support values ≥ 70% are in FIG. 1a, b.) Clades A and B are associated respectively to the Latin binomials *C. gloeosporioides* and *C. boninense* as evidenced by significant BLAST scores among sequences from taxa in these clades to ex-type sequences of *C. gloeosporioides* (EU371022; E value = 0, Max ident. = 98%; Cannon et al. 2008) and *C. boninense* (AB051400; E value = 0, Max. ident. = 100%; Moriwaki et al. 2003) respectively and by unpublished phylogenetic analyses. Clade A henceforth is referred to as the *C. gloeosporioides* clade and includes 55 of the 77 strains in the Panamanian collection and is the principal focus of this study. The identities of clades C and D could not be determined with the morphological and sequence data at hand. Apart from the use of sequences from clades C and D as phylogenetic outgroups the phylogenetics and taxonomy of clades B, C and D are not treated further here. The only lineage within the *C. gloeosporioides* clade that received significant bootstrap support was the terminal

clade consisting of strains isolated from anthracnose lesions on mango fruit (FIG. 1a). Because of the low informative variability of ITS among these members of the *C. gloeosporioides* clade ITS data were omitted from the multilocus dataset.

The 5′-*tef1* yielded concordant support for ITS clades A–D but differed from the ITS in delineating a greater number of distinct phylogenetic terminals within all four clades (FIG. 1a, b). In total 32 unique 5′-*tef1* haplotypes were distinguished and MP analysis resolved 18 distinct terminal-most branches or clades (FIG 1b). Within the *C. gloeosporioides* clade nine 5′-*tef1* haplotype subgroups were resolved and designated as OTUs. A total of 26 strains representing all nine *C. gloeosporioides* clade 5′-*tef1* haplotype groups were selected for multilocus sequencing.

*Multilocus phylogenetic analysis*.—Phylogenetic species boundaries and relationships among endophytic and pathogenic *C. gloeosporioides* clade subgroups diagnosed with 5′-*tef1* haplotype were inferred in a multilocus analysis of five nuclear genes that included 3′-*tef1*, *btub*, *apn2*, *apn2*/*mat*1igs and *rpb1*. Gapped positions in length-variable regions totaling 32, 181, 45 and 1 bp were excluded respectively from the β-*tub*, *apn2*, *apn2*/*Mat*1igs and *rpb1* data partitions. The aligned lengths of the individual 3′-*tef1*, β-*tub*, *apn2*, *apn2*/*Mat*1igs and *rpb1* data partitions, excluding gapped positions, were respectively 983, 1451, 756, 844 and 742 bp, totaling 4766 bp. The multilocus alignment was submitted to TreeBASE 10327; www.TreeBASE.org. Tree statistics from exploratory MP analyses of the individual partitions are summarized (TABLE I). MP BS analyses for the five individual partitions revealed strong gene-gene concordance (TABLE II) and a lack of topological discord between nodes that received > 70% bootstrap support. All analytical methods yielded identical tree topologies and the majority rule

consensus phylogram with branch lengths for the partitioned Bayesian analysis is presented (FIG. 3). Log-likelihood scores of the consensus BI and best ML trees were identical (−ln L 14324.21).

Nine terminal clades and branches were resolved in the multilocus analysis of the *C. gloeosporioides* clade (FIG. 3), each corresponding to the nine subgroups diagnosed with 5′-*tef1*: (i) the ex-epitype of *C. gloeosporioides* IMI 356878 (A1-1), which is nested within the *C. gloeosporioides* clade and is phylogenetically distinct from all Neotropical strains in this study; (ii) the strains isolated from diseased cacao fruit (A7-5), which are assigned to the species *C. theobromicola*, is the basal lineage of the *C. gloeosporioides* clade; (iii) the new species *C. ignotum*, a clade of endophytes of cacao and other plants, which included two shallow sister lineages that correspond to two 5′-*tef1* haplotype subgroups (A4-3, A5-4); (iv) *Colletotrichum* sp. indet. 1 (A6-4), a lineage isolated exclusively from lesions on ripe mango fruits that is being described elsewhere (T. Tarnowski pers comm); (v) *Colletotrichum* spp. indet. 2 and 3 (groups A8-6, A9-7), two clades of asymptomatic endophytes; and (vi) the new species *C. tropicale*, which includes two weakly supported sister clades that correspond to two 5′-tef1 subgroups (A2-1, A3-2), that is composed primarily of endophytic strains but also includes one strain isolated from lesions on mature fruit of guanábana (*Anona muricata)* and three strains isolated from lesions on mature leaves of avocado (*Persea americana)*. As defined here *C. ignotum* and *C. tropicale* each include two groups of strains with different 5′-*tef1* haplotype groups (FIGS. 1b, 3). In *C. ignotum* A5-4 and A4-3 groups are strongly supported as reciprocally monophyletic in the combined analysis (FIG. 3, TABLE II) and the majority of the individual data partitions provide support for one or the other or both

clades (TABLE II). By contrast support for the reciprocal monophyly of the *C. tropicale* A3-2 and A2-1 5′-*tef1* haplotype subgroups was not significantly supported by any single gene partition and only the A2-1 partition (E2303 + 8401) was supported in Bayesian and ML analysis of the combined dataset (FIG. 3). It is likely that the two 5′-*tef1* haplotype subgroups within both *C. tropicale* and *C. ignotum* represent phylogenetically distinct lineages. However additional evidence is needed to determine whether these differences warrant formal recognition.

*Chinese cacao endophytes*.—MP analysis of 5′-*tef1* sequences partitioned the 28 endophytic Chinese *Colletotrichum* strains into six distinct lineages within the *C. gloeosporioides* clade (FIG. 4; 10327; www.TreeBASE.org). Four subgroups were characterized by 5′-*tef1* intron configurations not observed among the Neotropical species, including subgroups A11-14, A12-15, A13-16, A15-17 (FIG. 2). The Chinese haplotype group A10-1 was identical in intron configurations with C. *tropicale* (A2-1) but formed a strongly supported sister clade of the former. The single Chinese strain A3-176 was nested within *Colletotrichum* sp. indet. 2 (A8-6) and differed by a single base pair.

*Host relationships of members of the C. gloeosporioides complex*.—The ex-epitype strain of *C. gloeosporioides*, which was isolated from a necrotic spot on a living leaf of *Citrus sinensis* in Italy (Cannon et al. 2008), was phylogenetically distinct from all Panamanian strains (FIGS. 1b, 3). Cacao leaf endophytes were distributed among four taxa including *C. tropicale* (A2-1, A3-2), *C. ignotum* (A4-3, A5-4), *Colletotrichum* sp. indet. 2 (A8-6), and *Colletotrichum* sp. indet. 3 (A9-7), with the largest group being *C. tropicale* (FIGS. 2b, 3). With the exception of *Colletotrichum* sp. indet. 2 (A8-6), which included

endophytes only from cacao (n = 5), each subgroup included endophytic strains isolated from cacao and from other tree species. *C. ignotum* also included strains isolated as endophytes from a species of *Zamia*, a member of Cycadales. In addition *Colletotrichum* sp. indet. 3, (A9-7), represented by the single strain G.J.S. 08-57, was isolated from asymptomatic leaves of *Persea americana*. The cacao endophytes *C. tropicale*, *C. ignotum* and *Colletotrichum* sp. indet 2 thus appear to be part of the background endophytic community in the Panamanian forest ecosystem. *Colletotrichum* sp. indet. 1 (A6-4) and *C. theobromicola* (A7-5) included strains isolated only from ripe or decaying fruit of mango and cacao respectively; they never were isolated as endophytes. Several cacao endophytes were not members of the *C. gloeosporioides* clade, including *C. boninense* 8975 (B1-8) and clade C strains 3386 (C5-11), 4766 (C2-9) and 4801 (C4-10). However strains of *C. boninense* and clade C also were isolated from other plant species. Clade D strains were isolated from two plant species other than cacao. Thus the *C. boninense* C and D clades also occur as nonspecific endophytes.

*Characterization of colonies and micromorphology in species recognized in* C. gloeosporioides *complex*.—*Colletotrichum* presents few morphological parameters that can be used to characterize species. We summarized the continuous and other phenotypic characters of the species discussed here (TABLE III). These characters include growth rate, colony appearance and degree of sporulation on artificial media, presence or absence of setae, conidial size and morphology and of phialides, and morphology of hyphal appressoria.

The optimum temperature for growth for most of the clades of the *C. gloeosporioides* complex was 25–30 C; for them colony diameter on PDA after 96 h was

44–53 mm. The exception was the mango clade, for which growth was much slower; the optimum temperature for growth on PDA was 25 C, and colony diameter was only ca. 20 mm. Most strains grew at 37 C, but none reached a diameter greater than 20 mm after 96 h on PDA. Strain G.J.S. 08-57 (FIG. 2b, subgroup A9-3), an endophyte of *Persea americana*, grew somewhat faster than all other strains that we studied (not shown).

The characteristics of colonies grown on PDA 10 d at 25 C under cool white fluorescent light/darkness in a 12 h cycle correlated to some extent with clades. (These differences can be seen in FIGS. 5–13.) The main differences were in the relative abundance of conidia, their distribution in the Petri plate and the overall colony color.

Conidiomata were formed abundantly in *C. gloeosporioides*, *C. tropicale* and members of the mango clade. *Colletotrichum gloeosporioides* produced abundant setae, but these were lacking or rare in all other clades. Although members of *C. ignotum* did not produce conidia on PDA or SNA with filter paper, conidiomata formed on pieces of sterilized carnation leaf scattered on the surface of SNA. Conidiomata formed sparingly in *C. theobromicola* (FIGS. 14, 15); dark hyphae radiated from the conidiomata into the agar (FIG. 15).

Phialides of all species were cylindrical or slightly tapered from base to tip, and phialide tips usually were marked by periclinal thickenings. There were no differences in dimensions of phialides among the clades. Differences were seen in proliferation of phialides. Sympodial proliferation of phialides was observed commonly in *C. ignotum* but was not seen in any of the other groups. In *C. theobromicola* phialides typically were produced from short-cell hyphae (FIGS. 16, 17). Percurrent proliferation of phialides was

not uncommon in the mango clade and in the *C. gloeosporioides* epitype, and multiple percurrent proliferations were particularly conspicuous in strain G.J.S. 08-57.

All members of the complex produce straight to slightly curved, subcylindrical conidia 12–20 × 4–6 µm. While there is considerable overlap in the conidial measurements when the mean plus and minus the standard deviation is considered, the 95% confidence intervals reveal significant differences among the clades (TABLE III). Thus conidia of *C. gloeosporioides* and *C. ignotum* are relatively short and wide and have a small L/W ratio while those of the members of *C. theobromicola* and especially the mango clade are long and narrow, thus having a larger L/W ratio. Conidia in G.J.S. 08-57 were unusually narrow, giving this strain a high L/W ratio.

Appressoria were present in all groups. In general they were subglobose to clavate and either not lobed or rarely lobed.

Perithecia were formed only in cultures of some members of *C. ignotum* (E183, E886). These perithecia were typical of *G. cingulata*, as this teleomorph is usually described (von Arx and Müller 1954).

Most of the phylogenetic species outside the *C. gloeosporioides* clade (i.e. not members of the *C. gloeosporioides* complex in FIG. 3) are morphologically inseparable from *C. gloeosporioides* on the basis of their subcylindrical, straight to slightly curved conidia and size. However clade B is identified as *C. boninense* (Moriwaki et al. 2003), a species originally isolated from a wide range of plants in Japan. It is also distinguished in part because of its wide conidia, which is consistent for our strains, although the L/W ratio of our strains is somewhat smaller than originally was reported for the species. In addition the sequence similarity and clustering of the ex type ITS1 sequence for *C.*

*boninense* (AB051400, Moriwaki et al. 2003) with clade B strains from this study (data not shown) helped confirm this species identification. Strains in clades C and D are not morphologically separable from the *C. gloeosporioides* clade or each other, although ITS evidence clearly distinguishes these three groups. Although clade C and D strain ITS sequences have close matches in GenBank, these accessions are identified either as *C. gloeosporioides* or are unidentified (data not shown), thus these clades might represent undescribed species.

TAXONOMY

Using a phylogenetic species concept based on genealogical concordance (Taylor et al. 2000) we recognize five species, *C. gloeosporioides*, *C. ignotum*, *C. tropicale* and *C. theobromicola*. The mango clade (FIG. 3, subgroup A6-4) is described elsewhere (T. Tarnowski pers comm). Subgroups A8-6 and A9-7 (FIG. 1b) represent two or more possibly undescribed species that are not treated here. Morphological and cultural character trends within clades of the *C. gloeosporioides* complex should not be considered as diagnostic; the exception is the slow growth of strains isolated from mango. Three species of *Colletotrichum* are described below. (Continuous measurements are reported in TABLE III.)

*Colletotrichum theobromicola* [as '*theobromicolum*' ] Delacr., Bull. Soc. Mycol. France 1905:191. 1905. FIGS. 5–7, 14–24

Optimum temperature for growth 25–30 C. Colonies on PDA 45–50 mm diam after 4 d at 25 C, at first light gray with hyaline immersed hyphae, after 2 or 3 d forming gray, dark concentric rings, entire colony becoming dense black with age; aerial mycelium dense light gray to gray. Conidiomata rudimentary, reduced to conidial masses, forming

in abundance in concentric rings, orange; setae not observed. On SNA the colonies nearly invisible; orange conidial mass dispersed through the colony on agar and from sterile filter paper. Conidiogenous cells cylindrical, tapering uniformly from base to tip, monoblastic, typically arising from highly septate, swollen hyphae on SNA. Conidia subcylindrical to clavate, often with broadly rounded ends, straight, sometimes developing a median septum in age. Swollen cells in the conidiomata not observed. Appressoria irregular, often somewhat lobed, (5–)6–10(–14) × (4–)5–6(–8) µm; terminal or lateral, dark brown, and sometimes proliferating to produce a second appressorium.

Habitat: Isolated from *Theobroma cacao* leaf and fruit lesions.

NEOTYPE: PANAMÁ. CHIRIQUI PROVINCE: Escobal, isolate from leaf spot of *Theobroma cacao*, Jan 2008, *E.I. Rojas* (BPI 879264, culture G.J.S. 08-50 = CBS 124945). Neotype designated herewith.

*Additional specimens examined:* PANAMÁ. CHIRIQUI PROVINCE: San Vicente, isolated from black lesion on pod of *Theobroma cacao*, 2008, *E.I. Rojas ER08-8* (BPI 879266, Culture G.J.S. 08-47 = CBS 125383); same collecting data, *E.I. Rojas ER08-9* (BPI 879265, Culture G.J.S. 08-48 = CBS 125393); same locality and date, collected from lesion on leaf of *Theobroma cacao*, *E.I. Rojas 08-10* (BPI 879267, Culture G.J.S. 08-49 = CBS 125384).

Notes. *Colletotrichum theobromicola* originally was isolated from fruit lesions of *Theobroma cacao* in the Antilles Françaises, or French West Indies, which include several islands in the Caribbean Sea (Delacroix 1905). The protolog is reasonably consistent with the collections that we have identified as *C. theobromicola*. We were not able to locate type material in PC or in MPA. Thus we assume the type to be nonexistent and accordingly designate a Panamanian collection as neotype with the ex neotype culture deposited in CBS.

**Colletotrichum tropicale** Rojas, Rehner & Samuels, sp. nov. FIGS. 8–10, 25–32

MycoBank MB515222

*Colletotricho gloeosporioidi (Penz.) Sacc*. simile sed setae absens et conidia majora, 12.5–16.5 × 4.7–5.5 µm mensa; endophyticum in foliis plantae vivae.

Holotypus BPI 879269.

Optimal temperature 25–30 C. Colonies 40–50 mm diam on PDA after 4 d at 25 C; aerial mycelium abundant to scant, white to light gray; no diffusing pigment observed; conidiomata forming abundantly in concentric rings, conidial masses slimy, orange. On SNA conidial masses abundant, dispersed through the colony and on filter paper. Conidiomata dark brown at base; setae rare. Conidiogenous cells cylindrical, monoblastic, tip with periclinal thickening, arising from a thin base of textura epidermoidea. Conidia subcylindrical with rounded ends, rarely clavate, straight, with or without a slightly protuberant, flat basal abscission scar. Swollen cells with granular contents and thin walls forming singly at tips of hyphal branches, (8.7–)10.2–12.7(–14.7) × (7.0–)8.2–11.2(–13.0) µm. Appressoria subglobose, clavate, fusiform; not lobed, terminal, (4.7–)7.0–11.0(–20.0) × (4.0–)5.2–7.2(–11.5) µm.

Habitat: Commonly isolated as an endophyte from leaves of a wide range of host species in tropical forests of Panamá, including *Theobroma cacao*, *Trichilia tuberculata*, *Viola surinamensis* and *Cordia aliodora*, also isolated from a rotting fruit of *Annona muricata*.

Etymology: "tropicale" refers to the known distribution of the species.

Known distribution: Panamá.

HOLOTYPE: PANAMÁ. BARRO COLORADO MONUMENT: isolated as an endophyte from a leaf of *Theobroma cacao*, *E.I. Rojas*, *L.C. Mejía & Z. Maynard 5101* (BPI 879269, culture 5101= CBS 124949). Holotype designated here.

Additional specimen examined: PANAMÁ. COLON PROVINCE: Nombre de Dios, isolated as an endophyte from a leaf of *Theobroma cacao*, *E.I. Rojas*, *L.C. Mejía & Z. Maynard 7423* (BPI 879268, Culture 7423).

**Colletotrichum ignotum** Rojas, Rehner & Samuels, sp. nov. FIGS. 11–13, 39–47

MycoBank MB515223

*Colletotricho gloeosporioidi* (Penz.) Sacc. simile sed endophyticum in foliis plantae vivae; conidia 13.5–17.5 × 4.5–5.7 µm mensa.

Holotypus: BPI 879262.

Colonies on PDA ca. 70 mm diam after 7 d at 25 C under light, off-white to slightly gray, orangish to gray in reverse; mycelium dense, colony sterile. Colonies on SNA nearly invisible, sterile after 10 d, conidiomata forming on sterile filter paper and on carnation leaf pieces under intermittent light; conidial masses pale orange; setae rarely observed. Phialides cylindrical or tapering slightly from base to tip, arising from a thin base of textura epidermoidea, tip with conspicuous periclinal thickening; proliferation not observed. Conidia subcylindrical with blunt ends to subfusiform (*3589*), lacking a visible basal abscission scar, remaining unicellular, subglobose to irregular, often slightly lobed, (5–)7–11(–17) × (4–)5–7(–10) µm. Swollen cells not observed. Appressoria clavate to fusiform, not lobed, terminal, (4.7–)7.0–11.0(–20.0) × (4.0–)5.2–7.2(–11.5) µm.

Etymology of the species epithet. "ignotum" refers to the cryptic, asymptomatic presence in leaves.

Habitat. Endophytic in leaves of dicotyledonous tropical plants including *Genipa americana*, *Tetragastris panamensis* and *Theobroma cacao*.

Known distribution. Panamá.

HOLOTYPE: PANAMÁ. BARRO COLORADO MONUMENT: Gigante, isolated as a leaf endophyte of *Tetragastris panamensis*, Jun 2004, *E.I. Rojas*, *L.C. Mejía & Z. Maynard* (BPI 879262, culture E886 = CBS 125397). Holotype designated here.

Additional specimens examined. PANAMÁ. Isolated as an endophyte from a leaf of *Theobroma cacao*, *E.I. Rojas*, *L.C. Mejía & Z. Maynard 3679* (BPI 879259, culture 3679 = CBS 125392); isolated as an endophyte from a leaf of *Theobroma cacao*, *E.I. Rojas*, *L.C. Mejía & Z. Maynard 1087* (BPI 879260,

culture 1087 = CBS 125391); isolated as an endophyte from a leaf of *Theobroma cacao*, *E.I. Rojas*, *L.C. Mejía & Z. Maynard 3589* (BPI 879261, culture 3589 = CBS 125390).

## DISCUSSION

The principal objectives of this study were to determine phylogenetic diversity among 77 Neotropical *Colletotrichum* strains associated as pathogens and asymptomatic endophytes of leaves of cacao and other plant species and to determine whether pathogenic and endophytic ecologies correlate with phylogeny. All strains conformed phenotypically to *C. gloeosporioides*, thus morphology was uninformative for categorizing these isolates. In contrast the combination of single and multilocus sequence data enabled the unique diagnosis of lineage diversity, definition of phylogenetic species limits and assessment of ecological mode within this morphologically cryptic taxon.

*Characterizing* Colletotrichum *genetic diversity*.—ITS and 5′-*tef1* sequences were used to appraise the phylogenetic and taxonomic diversity within the Panamanian collection, which in turn was used to develop an inclusive taxon-sampling scheme for the multilocus phylogenetic analysis of the *C. gloeosporioides* clade. The ITS locus was selected as a screening marker because of its widespread use for species discrimination in fungi. However because of the poor performance of ITS for species delimitation and identification in *Colletotrichum* and many other endophytic ascomycete fungi (Arnold et al. 2007, Arnold and Lutzoni 2007, Gallery et al. 2007, Higgins et al. 2007, Crouch et al. 2009a, U'Ren et al. 2009) we sought to identify a more informative marker for screening diversity within environmental samples of *Colletotrichum*. The 5′-*tef1* region was selected for evaluation because of its use in speciation studies in other sordariaceous fungi, including *Fusarium* (Geiser et al. 2004), *Trichoderma* (Druzhinina et al. 2005) and

*Metarhizium* (Bischoff et al. 2009), and because this genomic region was determined to be highly variable in *Colletotrichum* at an early stage of this investigation.

In this study analyses of ITS and 5′-*tef1* (FIG. 1a, b) concordantly resolved four supported clades, corresponding to the *C. gloeosporioides* and *C. boninense* clades, and two taxonomically undetermined clades, C and D. Apart from these points of topological agreement, the 5′-*tef1* was distinctly more informative than ITS in detailing terminal diversity across clades A–D. This is evidenced in the 18 terminal-most branches and clades that received bootstrap support ≥ 70% and/or displayed ≥ 5% sequence dissimilarity from sister lineages (FIG 1a, b; SUPPLEMENTAL TABLE III). The diagnostic utility of 5′-*tef1* was demonstrated clearly in the case of the *C. gloeosporioides* clade (FIG. 1b), where all nine distinct 5′-*tef1* haplotypes were shown to correspond to phylogenetic terminals diagnosed in the multilocus phylogenetic analysis (TABLE II, FIG. 3). In contrast ITS provided significantly less phylogenetic resolution within all four major clades, including the *C. gloeosporioides* clade (FIG. 1a), where it failed to distinguish clearly among phylogenetic species isolated as asymptomatic endophytes. This also is reflected by the short ITS genetic distances among *C. gloeosporioides* clade taxa, which are at or below 1% (SUPPLEMENTAL TABLE III). Although results presented here demonstrate the limitations of ITS for resolving closely related phylogenetic species in the *C. gloeosporioides* complex, ITS retains value as a taxonomic reference marker in *Colletotrichum* because of its wide usage in fungal ecology and systematics and for its ability to distinguish among deeper lineages, as demonstrated by Cai et al. (2009). However the relatively poor performance of ITS in species diagnosis and phylogenetic reconstruction among closely related *Colletotrichum* species examined here indicate that

more innately variable loci such as 5′-*tef1* are more effective for species diagnosis and biodiversity screening.

The unusual capacity of 5′-*tef1* to consistently distinguish among low-level phylogenetic terminals in *Colletotrichum* stems principally from sequence differences accumulating at 10 spliceosomal intron sites, nine at which introns are variably present or absent among the taxa examined in this study (FIG. 2). Molecular diversity at 5′-*tef1* therefore accrues as a combination of the gain and loss of introns (turnover) and sequence divergence among homologous introns. Intron turnover at intron positions 1–5 thus has led to the creation of mutually exclusive intron arrays at a minimum of one to as many as three intron positions among different intron configuration types (FIG. 2). It is the presence or absence of such introns that help to account for the distinctive genetic distances among taxa with different configuration types (SUPPLEMENTAL TABLE III). However it is apparent that some syntopic introns are homoplastic (e.g. introns 1, 2.2, 2.3, 4.1–4.3, 5; FIG. 2), as indicated by the juxtaposition of highly divergent sequences at these particular intron positions. This apparent homoplasy could be due to saturating levels of sequence evolution between intron homologs, intron-sliding events that shift adjacent, non-homologous introns into syntopic alignment, or the result of independent intron gains. Regardless of origin the forced alignment of divergent, homoplastic introns constitutes a potential source of error for phylogenetic reconstruction methods due to long-branch attraction (LBA; Bergsten 2005) and provides a possible explanation for the internal topological differences between the 5′-*tef1* topology (FIG. 1b) and the multilocus phylogeny of the *C. gloeosporiodes* clade (FIG. 3). For these reasons we limit our use of 5′-*tef1* to diagnose terminal diversity and caution against its use for phylogenetic

inference, particularly when divergent syntopic introns are evident in the sequence alignment.

A 5′-*tef1* diversity screen of Chinese *Colletotrichum* endophyte strains isolated from cacao resolved six *C. gloeosporioides* taxa (FIG. 4). A single Chinese isolate, A3-176, grouped with the Neotropical *Colletotrichum* sp. indet. 2. The sequence similarity between these Asian and Neotropical strains could be attributed either to a lack of divergence between disjunct populations or due to recent introduction or intercontinental dispersal of this lineage. *Colletotrichum* has been isolated from the seed coats of *Cecropia* germinated in Neotropical soils (U'ren et al. 2009), and it is possible that A3-176 is descended from infected seeds used to establish these plants in China (Van Bael pers obs). The five additional Chinese subgroups differ from Neotropical taxa in having different 5′-*tef1* intron organizations (FIG. 2) and might represent distinct Asian species. This finding is consistent with the prediction of Arnold and Lutzoni (2007) that species assemblages of commonly occurring genera of endophytes such as *Colletotrichum* are likely to vary among biomes.

Evolutionary turnover and sequence divergence among 5′-*tef1* introns in *Colletotrichum* have been shown here to be highly dynamic and evidently are closely scaled to lineage diversification in this genus. Given the predictive accuracy of 5′-*tef1* as a leading indicator of phylogenetic speciation in *Colletotrichum*, this short divergent sequence should be similarly useful for routine species identification, particularly as sequences from authenticated ex type strains are made available in publicly accessible databases. Because sequence divergence at 5′-*tef1* in *Colletotrichum* typically exceeds 5% among closely related phylogenetic species accurate and unambiguous species

identification with 5′-*tef1* should be possible with widely available BLAST sequence similarity query strategies.

*Species concepts in the* C. gloeosporioides *clade*.—We evaluated the utility of multilocus sequence data, morphology and host association as characters for the diagnosis of species in the *C. gloeosporioides* morphospecies complex. Only the molecular characters were consistent in uniquely delineating and differentiating among all taxa considered here. Three loci in particular, including *btub*, a marker that is frequently used in fungal phylogenetics, and the nuclease *apn*2 and the adjacent intergenic spacer *apn*2/*mat*12igs, which were described recently for phylogenetics of falcate-spored graminicolous species of *Colletotrichum* by Crouch et al. (2009a, b, c), individually provided robust support for species boundaries and relationships within the *C. gloeosporioides* clade (TABLE II). In addition exon regions of *btub* and *apn*2 are highly informative of relationships among diverse *Colletotrichum* taxa (Rehner, Rojas and Samuels unpubl) and thus are promising candidate loci for use in more inclusive molecular phylogenetic analyses of the genus.

In contrast to unambiguous species diagnoses achieved with molecular data, we were unable to identify any phenotypic features, whether conidia, hyphal appressoria or colony growth characteristics, that differentiated among *C. gloeosporioides* clade pathogens, endophytes or clades resolved in this study. A similar conclusion about the inadequacy of morphology for *Colletotrichum* systematics was rendered by Sutton (1992) without benefit of molecular data. Sutton's assessment has been confirmed by more recent molecular studies demonstrating that several traditional morphological species in *Colletotrichum* are complexes of morphologically indistinguishable phylogenetic species (Du et al. 2005, Crouch et al. 2009c). Nonetheless diagnostic morphological features

have been described for several recently described species of *Colletotrichum* (Crouch et al. 2009c, Yang et al. 2009), although recognition of these features was aided by knowledge of phylogenetic species boundaries.

Host association heretofore has been the principal criterion used for species recognition and identification in *Colletotrichum*, and this single attribute accounts for the many species names proposed in the genus. The majority of *Colletotrichum* strains in this study were isolated as asymptomatic endophytes from more than one plant host species. Consequently host association is uninformative for the identification of these species. In contrast both *C. theobromicola* and *Colletotrichum* sp. indet. 1 appear to be respectively specific pathogens of cacao and mango. However host range data for these *Colletotrichum* species, as well as the characterization of other *Colletotrichum* species associated with cacao and mango, is needed to objectively assess the utility of host association as a method for diagnosis of these *Colletotrichum* species.

Taken together species boundaries in the *C. gloeosporioides* clade are best resolved with a phylogenetic species concept based on the concordance of multiple genes (Taylor et al. 2000). While morphology, host association and ecological mode may prove useful in the identification of some *Colletotrichum* species, definitive species diagnosis and identification for most species in the *C. gloeosporioides* clade is practicable only with phylogenetically informative molecular characters.

*Ecological mode of* Colletotrichum *species*.—One objective of this study was to determine whether endophytic strains of *Colletotrichum* represent evolutionary lineages distinct from those of pathogenic strains. The main hope being that it would be safe to use endophytic strains of what at the time was identified as *C. gloeosporioides* (Arnold et

al. 2003, Herre et al. 2007, Mejía et al. 2008) in the control of cacao diseases. The molecular phylogenetic analysis gave an interesting if equivocal result, the interpretation of which depends upon the definition of the word pathogen. Two clades included only strains that were isolated from lesions on fruits or leaves, namely *C. theobromicola* and *Colletotrichum* sp. indet. 1 (FIG. 3, *Colletotrichum* sp. indet., 5′-*tef1* haplotype subgroup A6-4); members of both these clades were distinct from strains isolated from asymptomatic plant tissue (i.e. endophytes). By contrast most of the asymptomatic endophytic strains clustered in clades that did not include pathogens. Of note, the endophyte clades typically included strains also isolated from native Panamanian trees unrelated to cacao. Whereas "endophytic" *Colletotrichum* strains were isolated from multiple host species, "pathogenic" strains appeared to be limited to single host species. The lack of host specificity in endophytic colonization was noted by Lu et al. (2004) in their study of endophytic *Colletotrichum* strains from a forest in Guyana.

The multilocus analysis that we have presented reveals an apparent dichotomy between strains isolated from diseased plant material, fruit or leaves, and those isolated from asymptomatic leaves. In the present work most of the endophytic strains isolated from cacao and other plant species are *C. tropicale*. This is apparently a common endophytic species in the diverse Panamanian forest, occurring asymptomatically in a wide range of hosts. However three strains of this species were isolated from lesions on old leaves of *Persea americana* and one was isolated from ripe rot of a fruit of *Annona muricata*.

Is it safe then to use "endophytic" *Colletotrichum* species as agents of biological control in cacao? Our data suggest that some species, such as *C.*

*theobromicola*, are capable of causing anthracnose in leaves, but we did not detect these species causing asymptomatic infections of cacao leaves. Obviously this species cannot be recommended for biological control applications. Our data also suggest that some species, such as *C. ignotum* and *C. tropicale*, might asymptomatically infect leaves and fruit of cacao but for unknown reasons these species do not induce symptoms in leaves; whereas changes in the inter- and intracellular environment that occur during fruit ripening, including the break down of preformed antifungal substances present in unripe fruits (Prusky and Lichter 2007, Munch et al. 2008), might enable these "endophytic" *Colletotrichum* species to actively colonize fruits, resulting in ripe rot. If this is actually the case then, despite the experimental evidence that some "endophytic" *Colletotrichum* species may protect cacao trees from disease (Mejia et al 2008, Arnold et al 2003), application of inoculum such that fruits of other plant species also are exposed to infection could lead to increased postharvest losses. However additional information on the ecology and epidemiology of fruit-rotting *Colletotrichum* species is needed to more clearly define this potential risk.

*In conclusion*.—Molecular diagnostic and genealogical exclusivity have been essential in providing novel insights into the diversity and ecology of *Colletotrichum* species associated as endophytes and pathogens of cacao and other Neotropical plants. In particular the discovery of 5′-*tef1* intron sequences as a proximate method for species diagnosis shows potential to enhance the speed and efficiency of discovery of *Colletotrichum* biodiversity. In Panamá the *C. gloeosporioides* morphospecies consists of four phylogenetically distinct groups, although their precise relationship to one another requires a more inclusive analysis of the genus. Of these four lineages the *C.*

*gloeosporioides* clade was the most abundant in endophyte surveys and consists of multiple phylogenetic species lineages whose ecologies can be characterized generally as pathogenic or endophytic. Continued investigation of the C. *gloeosporioides* clade will seek to clarify the basis of pathogenicity and endophytism and the evolutionary and ecological relationship of these distinctive ecological modes.

ACKNOWLEDGMENTS

We are sincerely grateful for the help of Veronica Martins, Clare Whittaker and Adnan Ismaiel who provided invaluable laboratory support throughout this study. For help in the field and lab in Panamá we thank Luis Ramirez, Dora Alvarez, Zuleyka Maynard, Luis C. Mejía, Terri Shirshac, Jill Urriola, Clara Perez, Luciano Arauz, Kenman Concepción, Mirna Santana and Rohan Hebbar. We thank Kaoru Kitajima and S. Joseph Wright for letting us find samples in their common-garden plots and the Autoridad Nacional del Ambiente de Panamá for permitting collection and exportation of samples. The mention of trade products or company names or firm names does not imply that the U.S. Department of Agriculture recommends them over similar products or companies not mentioned.

LITERATURE CITED

Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R. 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99:185–206.

———, Lutzoni F. 2007. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology 88:542–549.

———, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100:15649–15654.

Bailey JA, Jeger MJ. 1992. *Colletotrichum*: biology, pathology and control. Wallingford, UK: CAB International. 388 p.

Bergsten J. 2005. A review of long-branch attraction. Cladistics 21:163-193.

Bischoff JD, Humber RA, Rehner SA. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101:508–528.

Cannon PF, Buddie AG, Bridge PD. 2008. The typification of *Colletotrichum gloeosporioides*. Mycotaxon 104:189–204.

———, Simmons CM. 2002. Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. Mycologia 94:210–220.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556.

Croat TB. 1978. Flora of Barro Colorado Island. Stanford, California: Stanford Univ. Press. 943 p.

Crouch JA, Clarke BB, Hillman BI. 2006. Unraveling evolutionary relationships among the divergent lineages of *Colletotrichum* causing anthracnose in turfgrass and corn. Phytopathology 96:46–60.

———, ———, ———. 2009a. What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group. Mycologia 101:648–656.

———, Tredway LP, Clarke BB, Hillman BJ. 2009b. Phylogenetic and population divergence correspond with habitat for the pathogen *Colletotrichum cereale* and allied taxa across diverse grass communities. Mol Ecol 18:123–135.

———, Clarke GG, White JD Jr, Hillman BI. 2009c. Systematic analysis of the falcate-spored graminicolous *Colletotrichum* and a description of six new species from warm season grasses. Mycologia 101:717–732.

Damm U, Woudenberg JHC, Cannon PF, Crous PW. 2009. *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Divers 39:45–87.

Delacroix G. 1905. Travaux de la Station de Pathologie Vegetale II: Champignons parasites de plantes cultives dans les regions chaudes. Bull Soc Mycol Fr 191–192.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 1991. Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008.

Druzhinina IS, Kopchinskiy AG, Komon M, Bissett J, Szakacs G, Kubicek CP. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet Biol 42:813–8222.

Du M, Schardl CL, Nuckles EM, Vaillancourt LJ. 2005. Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. Mycologia 97:641–658.

Duran EL, Ploper LD, Ramallo JC, Grandi RAP, Giancoli C, Azevedo JL. 2005. The foliar fungal endophytes of *Citrus limon* in Argentina. Can J Bot 83:350–355.

Farr DF, Aime MC, Rossman AY, Palm ME. 2006. Species of *Colletotrichum* on Agavaceae. Mycol Res 110:1395–1408.

———, Rossman AY. 2009. Fungal Databases, Systematic mycology and Microbiology Laboratory, ARS, USDA. Retrieved 15 Sep 2009, from http://nt.ars-grin.gov/fungaldatabases/

Gallery RE, Dalling JW, Arnold AE. 2007. Diversity, host affinity and distribution of seed-infecting fungi: a case study with *Cecropia*. Ecology 88:582–588.

Gamboa MA, Bayman P. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). Biotropica 33:352–360.

Gams W, van der Aa HA, van der Plaats-Niterink AJ, Samson RA, Stalpers JA. 1987. CBS course of mycology. 3rd ed. Baarn, Netherlands: Centraalbureau voor Schimmelcultures.

Geiser DM, Jiminez-Gasco MdelM, Kang SC, Makalowsk, I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K. 2004. FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. Eur J Plant Pathol 110:473–479.

Guerber JC, Bo L, Johnston PR, Correll J.C. 2003. Characterization of diversity of *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs and mating compatibility. Mycologia 95:872–895.

Herre EA, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Butler A, Van Bael S. 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88:50 –558.

Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD, Lutzoni F. 2007. Phylogenetic relationships, host affinity and geographic structure of boreal and arctic endophytes from three major plant lineages. Mol Phylogen Evol 42:543–555.

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17:754–755.

Johnston PR, Jones D. 1997. Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia 89:420–430.

Katoh, K, Kuma K-I, Toh H, Miyata T. 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518.

———, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 94:286–298.

Liu B, Wasilwa LA, Guerber JC, Morelock TE, O'Neill NR, Correll JC. 2007. Comparison of *Colletotrichum orbiculare* and allied *Colletotrichum* species for mtDNA RFLPs, intron RFLPs, sequence variation, vegetative compatibility and host specificity. Phytopathology 97:1305–1314.

Lodge DJ, Fisher PJ, Sutton BC. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. Mycologia 88:733–738.

Lu G, Cannon PD, Reid A, Simmons CM. 2004. Diversity and molecular relationships of endophytic *Colletotrichum* isolates from Iwokrama Forest Reserve, Guyana. Mycol Res 108:53–63.

Matheny PB, Liu Y-J, Ammirati JF, Hall BD. 2002. Using RPB1 to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). Am J Bot 89:688–698.

Mejía LC, Enith I. Rojas EI, Maynard Z, Van Bael S, Arnold AE, Hebbar P, Samuels GJ, Robbins N, Herre EA. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol Control 46:4–14.

Mills PR, Hodson A, Brown AE. 1992. Molecular differentiation of *Colletotrichum gloeosporioides* isolates infecting tropical fruits. In: Bailey JA, Jeger MJ, eds. *Colletotrichum*: biology, pathology and control. Wallingford, UK: CAB International. p 269–288.

Morakotkarn D, Kawasaki H, Seki T. 2007. Molecular diversity of bamboo-associated fungi isolated from Japan. FEMS Microbiol Lett 266:10–19.

Moriwaki J, Sato T, Tsukiboshi T. 2003. Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan. Mycoscience 44:753.

Munch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB. 2008. The hemibiotrophic lifestyle of *Colletotrichum* species. Plant Physiol 165: 4151.

Nirenberg HI. 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. Mitteilungen aus der Biologischen Bundesanstalt für Land-und Forstwirtschaft Berlin-Dahlem 169:1-117.

Nylander JAA. 2004. MrModeltest. Version 2. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala Univ. Program distributed by the author.

Osono T. 2008. Endophytic and epiphytic phyllosphere fungi of *Camillea japonica*: seasonal and leaf-dependent variations. Mycologia 100:387–391.

Petrini O, Petrini LE, Rodrigues K.1995. Xylariaceaous endophytes: an exercise in biodiversity. Fitopatol Brasil 20:531–539

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

Prihastuti H, Cai L, Chan H, McKenzie EHC, Hyde KD. 2009. Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Divers 39:89–109.

Prusky D, Lichter A. 2007. Activation of quiescent infections by postharvest pathogens during transition from the biotrophic to the necrotrophic stage. FEMS Microbiol Lett 268:1–8.

Rehner SA, Buckley EP. 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97:84–98.

Rogozin IB, Lyons-Weller J, Koonin EV. 2000. Intron sliding in conserved gene families. Trends Genet 16:430–432.

Rojas EI, Herre EA, Mejía LC, Arnold AE, Chaverri P, Samuels GJ. 2008. *Endomelanconiopsis*, a new anamorph genus in the Botryosphaeriaceae. Mycologia 100:760–775.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Samuels GJ, Petrini O, Müller E. 1979. *Holmiella sabinae* (de Not.) comb. nov. (syn. *Eutryblidiella sabinae*) and its *Cornicullariella*-like anamorph. Ber Schweiz Bot Ges 89:80–91.

Sieber TN. 2007. Endophytic fungi in forest trees: Are they mutualists? Fungal Biol Rev 21:75–89.

Stone JK, Bacon CW, White JF Jr. 2000. An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF, eds. Microbial endophytes. New York: Marcel Dekker. p 3–29.

Sutton BC. 1980. The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Kew, UK: Commonwealth Mycological Institute.

———. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. In: Bailey JA, Jeger MJ, eds. *Colletotrichum*: biology, pathology and control. Wallingford, UK: CAB International. p 1–26.

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett D S, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32.

U'Ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE. 2009. Diversity and evolutionary origins of fungi associated with seeds of a Neotropical pioneer tree: a case study for analyzing fungal environmental samples. Mycol Res 113:432–449.

von Arx JA. 1957. Die Arten der Gattung *Colletotrichum* Corda. Phytopathol Z 29:413–468.

———, Müller E. 1954. Die Gattungen der amerosporen Pyrenomyceten. Beitr Kryptogamenfl Schweiz 11(1):1434.

Wattad C, Freeman S, Dinoor A, Prusky D. 1995. A nonpathogenic mutant of *Colletotrichum magna* is deficient in extracellular secretion of pectate lyase. Mol Plant-Microbe Interact 8:621–626.

Whalley AJS. 1996. The xylariaceaous way of life. Mycol Res 100:897–922.

White TJ, Bruns TD, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: Academic Press. p 315–322.

Wilkinson L. 2000. SYSTAT 10. Statistics I. Chicago: SPSS.

Yang YL, Liu ZY, Cai L, Hyde KD, Yu ZN, McKenzie EHC. 2009. *Colletotrichum* anthracnose of Amaryllidaceae. Fungal Divers 39:123–146.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [Doctoral dissertation]. Austin: Univ. Texas Press. 115 p.

## LEGENDS

FIG. 1. Maximum parsimony trees of (a) ITS and (b) 5′- *tef1* used to assess phylogenetic diversity within the Panamanian *Colletotrichum* collection. Bootstrap support > 70% is indicated adjacent to relevant nodes. Unique terminal branches and clusters (b) are labeled according to 5′-*tef1* haplotype subgroupings (diagramed in FIG. 2). Asymptomatic endophyte strains and symptomatic (pathogen) isolates are labeled respectively in plain and bold fonts. Both ITS and 5′-*tef1* trees are rooted with sequences from clade D.

FIG. 2. 5′-*tef1* intron arrangements in *Colletotrichum* (types 1–16). Top line: intron insertion locations within the 5′-tef1 exon are numbered consecutively in 5′ to 3′ order of occurrence. Lines 2–17: schematic map of intron configuration types; 5′-*tef1* subgroup codes for Panamanian and Chinese *Colletotrichum* strains are listed to the right of each map and consist of a three-part alphanumeric code denoting ITS clade (A–D), 5′-*tef1* subgroup (1, 2, 3…), "-" and intron configuration type.

FIG. 3. Bayesian majority rule consensus phylogram with branch lengths for the combined five-gene analysis of the *C. gloeosporioides* clade. Clades are identified to species, where applicable, and/or according to 5′-*tef1* group codes (listed in FIGS. 1b, 2). Support values exceeding 70%, 95% and 70% are shown above branches for MP, BI and ML analyses respectively or "-" where support was less than these specified threshold values. The analysis was rooted with sequences from clade C.

FIG. 4. 5′-*tef1* maximum parsimony tree of 28 Chinese and selected Panamanian *C. gloeosporioides* clade strains. Chinese strains are highlighted in boldface, and clades are labeled according 5′-*tef1* haplotype group. *C. theobromicola* was used to root the analysis.

FIGS. 5–13. Colonies of *Colletotrichum* species at 9 d grown at 25 C under 12 h darkness/12 h cool white fluorescent light on PDA. 5–7. *C. theobromicola*. 5. G.J.S. 08-48. 6. G.J.S. 08-43. 7. G.J.S. 08-50. 8–10.

*C. tropicale*. 8. 7423. 9. 5101. 10. Q633. 11–13. *C. ignotum*. 11. 1087. 12. E183. 13. *C. gloeosporioides* IMI356878.

FIGS. 14–24. *Colletotrichum theobromicola*. 14. Conidiomata on *T. cacao* fruit segments and SNA, G.J.S. 08-48. 15. Conidiomata on SNA, G.J.S. 08-43. 16–18 Conidiogenous cells on SNA. 16, 17. Phialides developed from septate, swollen hyphae. 16 G.J.S. 08-50. 17. G.J.S. 08-43. 18, 19. Phialides from acervulii. 18. G.J.S. 08-48. 19. G.J.S. 08-43. 20. Conidia, subcylindrical to subclavate (G.J.S. 08-50). 21. Conidia, subcylindrical to clavate (G.J.S. 08-48). 22, 23. Appressoria, SNA. 22. Multilobed appressoria, G.J.S. 08-48. 23. Appressoria proliferation, G.J.S. 08-50. 24. Cacao fruit with dark anthracnose lesions from which *C. theobromicola* was isolated. Bars: 14 = 0.5 mm, 15 = 0.25 mm, 16–23 = 20 µm.

FIGS. 25–38. *Colletotrichum tropicale*. 25. Slimy orange conidiomata, SNA, 7423. 26. Conidiogenous cells, SNA, Q633. 27, 28. Swollen cells or globose conidia, SNA, 5101. 29, 30. Conidia, SNA, Q633, 7423. 31, 32. Appressoria, SNA, 7423, 5101. 33–38. *Colletotrichum gloeosporioides*, IMI 356878. 33. Black conidiomata with abundant black hairs, SNA. 34. Dark brown setae. 35. Conidiogenous cells. 36. Conidia. 37, 38. Appressoria. Bars: 25, 33 = 0.5 mm; 26–29 = 20 µm; 30 = 10 µm; 31, 32, 34–38 = 20 µm.

FIGS. 39–47. *Colletotrichum ignotum*. 39, 40. Conidiogenous cells on SNA, 3679. 41. Subcylindrical conidia with blunt ends, E886. 42. Appressoria subglobose to irregular, some slightly lobed, 1087. 43. Setae, 7574. 44. Perithecia, E886. 45, 46. Asci, 7574, E886. 47. Ascospores, E183. Bars: 39–42, 46, 46 = 10 µm; 44, 47 = 100 µm.

## FOOTNOTES

Submitted 30 Sep 2009; accepted for publication 30 Mar 2010.

[1]Corresponding author. E-mail: stephen.rehner@ars.usda.gov

TABLE I. Character and MP tree statistics for ITS, *5′-tef1*, *apn2*, *btub*, *3′-tef1*, *rpb1*, *apn2*/MAT12igs

| Locus | # Characters | # MPTs [a] | MPT Length | CI [b] | RI [c] | #PIC (PIC/bp) [d] |
|---|---|---|---|---|---|---|
| *ITS* [e] | 576 | 22 | 120 | 0.808 | 0.771 | 83 (14.4) |
| *5′-tef1* [e] | 709 | 2 | 723 | 0.621 | 0.5606 | 255 (36) |
| *apn2* [f] | 724 | 8 | 295 | 0.875 | 0.823 | 176 (24.3) |
| *btub* [f] | 1451 | 1 | 352 | 0.926 | 0.893 | 245 (16.8) |
| *3′-tef1* [f] | 983 | 1 | 57 | 0.825 | 0.768 | 47 (4.8) |
| *rpb1* [f] | 742 | 8 | 187 | 0.920 | 0.880 | 149 (20.1) |
| *apn2/MAT12igs* [f] | 844 | 1 | 619 | 0.876 | 0.833 | 430 (50.9) |
| Combined [f] | 3461 | 2 | 1444 | 0.875 | 0.830 | 1056 (30.5) |

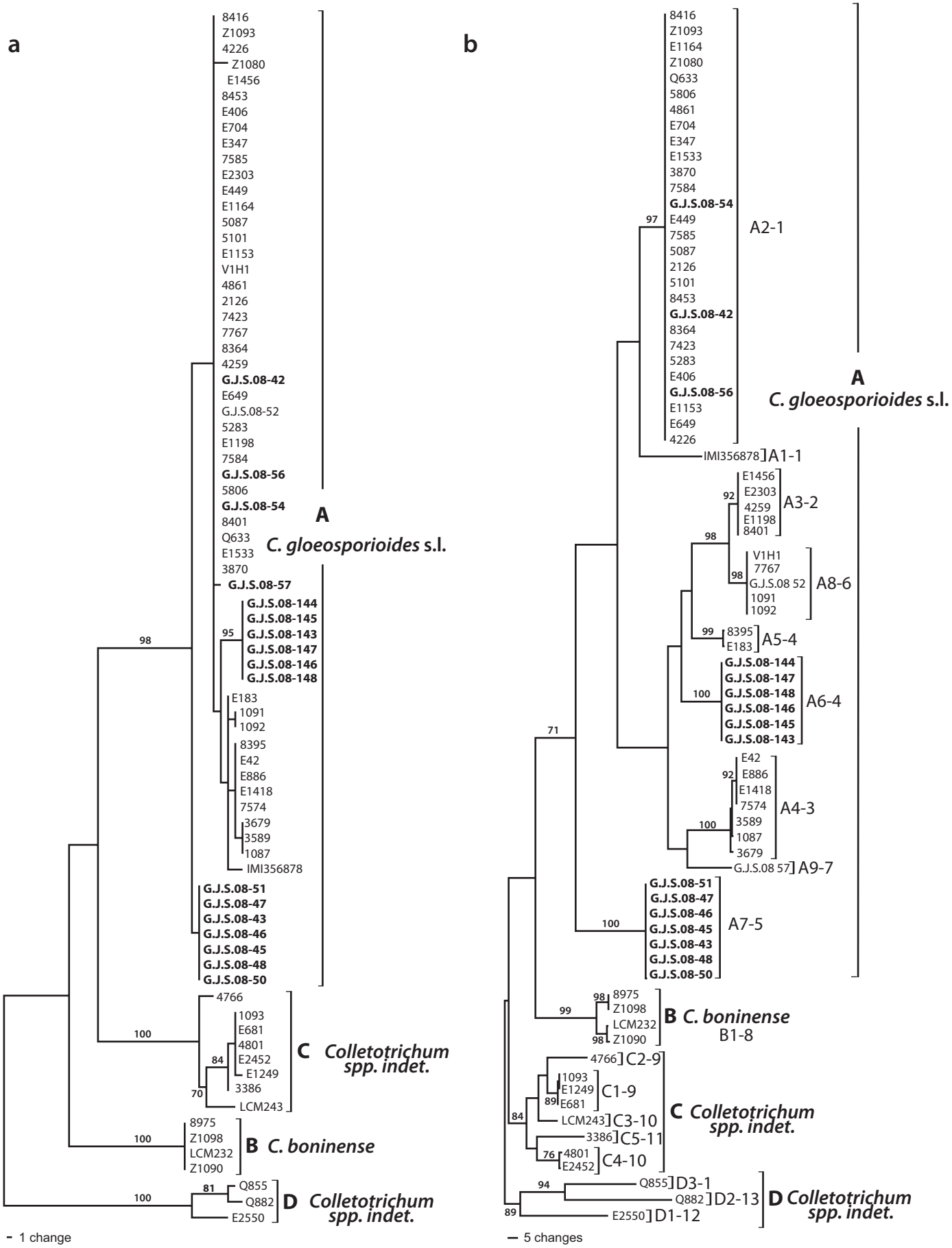
[a] MPTs, most parsimonious trees.
[b] CI, consistency index.
[c] RI, retention index.
[d] PIC/bp, per cent parsimony informative characters/ total base pairs.
[e] 77-taxon screening dataset.
[f] Loci in 29-taxon multilocus dataset.

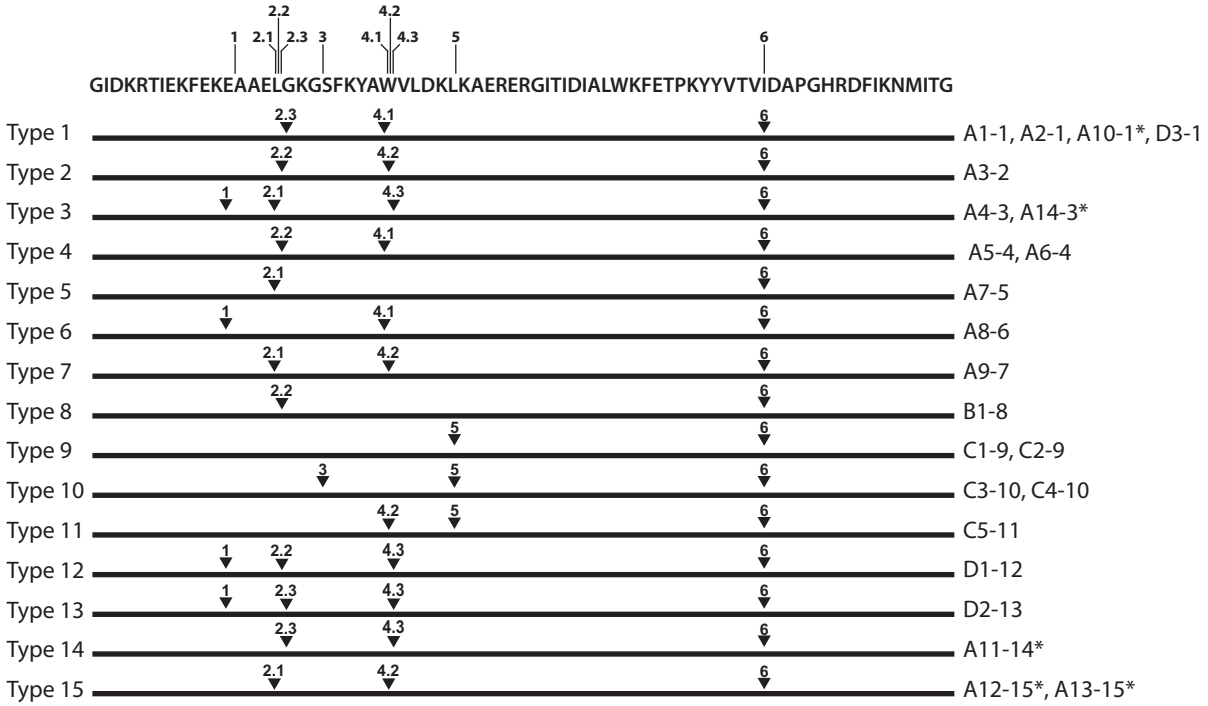
TABLE II. MP bootstrap support (> 50%) from individual and combined gene partitions for phylogenetic species in 29-taxon *C. gloeosporioides* clade analysis

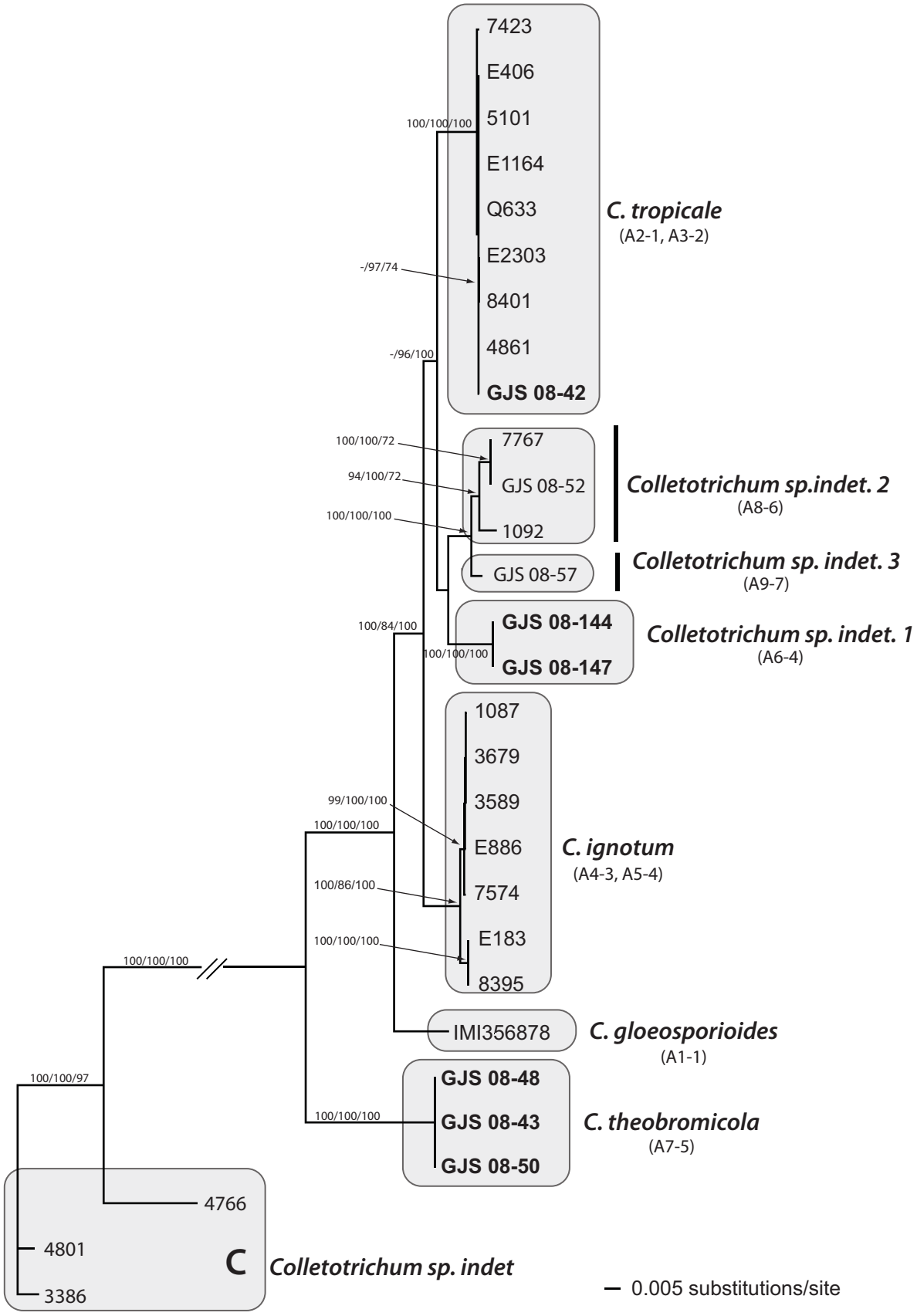
| Species/subgroup | 3′-*tef1* | *Btub* | *rpb1* | *apn2* | *apn2*/MAT12igs | Combined |
|---|---|---|---|---|---|---|
| *C. tropicale* (A2-3 + A3-3) | 60 | 100 | 73 | 100 | 100 | 100 |
| *C. tropicale* (A2-3) | – | – | – | – | – | 61 |
| *C. tropicale* (A3-3) | 51 | – | – | – | – | 67 |
| *Colletotrichum* sp. indet. (A8-2) | 51 | 95 | – | 99 | 99 | 94 |
| *Colletotrichum* sp. indet. (A9-3) | – | 86/95 | – | – | 100/99 | NA |
| *Colletotrichum* sp. indet.( A6-3) | 98 | 100 | 99 | 100 | 100 | 100 |
| *C. ignotum* (*A4-1* + *A5-3*) | – | 100 | 62 | 99 | 100 | 100 |
| *C. ignotum* (A4-1) | 87 | - | 64 | 99 | 87 | 99 |
| *C. ignotum* (A5-3) | – | 100 | 60 | 100 | 100/87 | 100 |
| *C. gloeosporioides* (A1-3) | NA | NA | NA | NA | NA | NA |
| *C. theobromicola* (A7-6) | 99 | 100 | 100 | 100 | 100 | 100 |

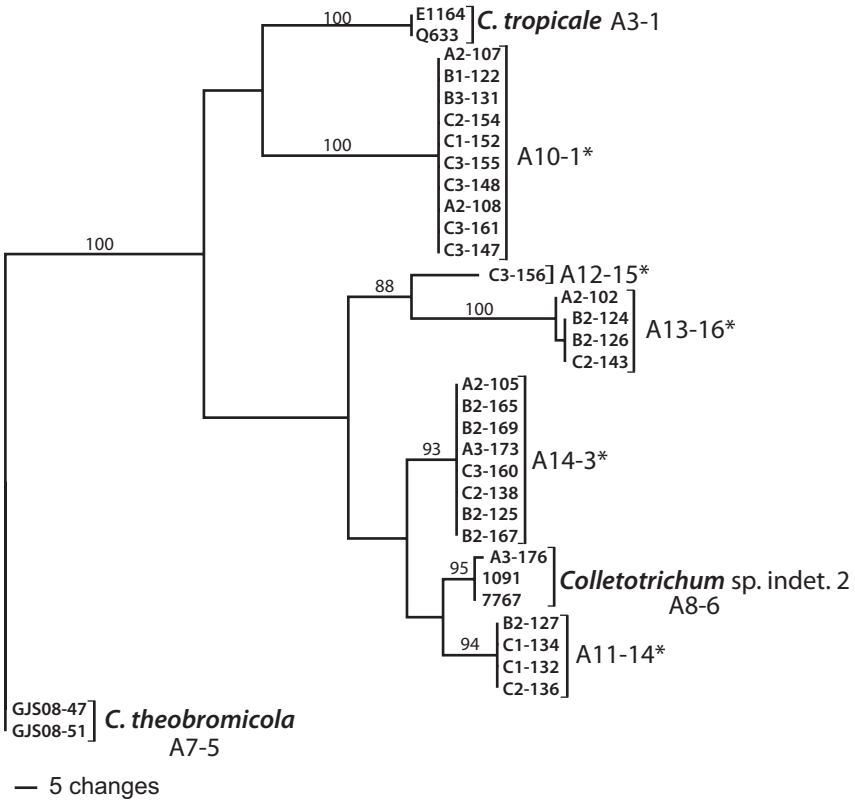
TABLE III. Characters of members of the *Colletotrichum gloeosporioides* complex

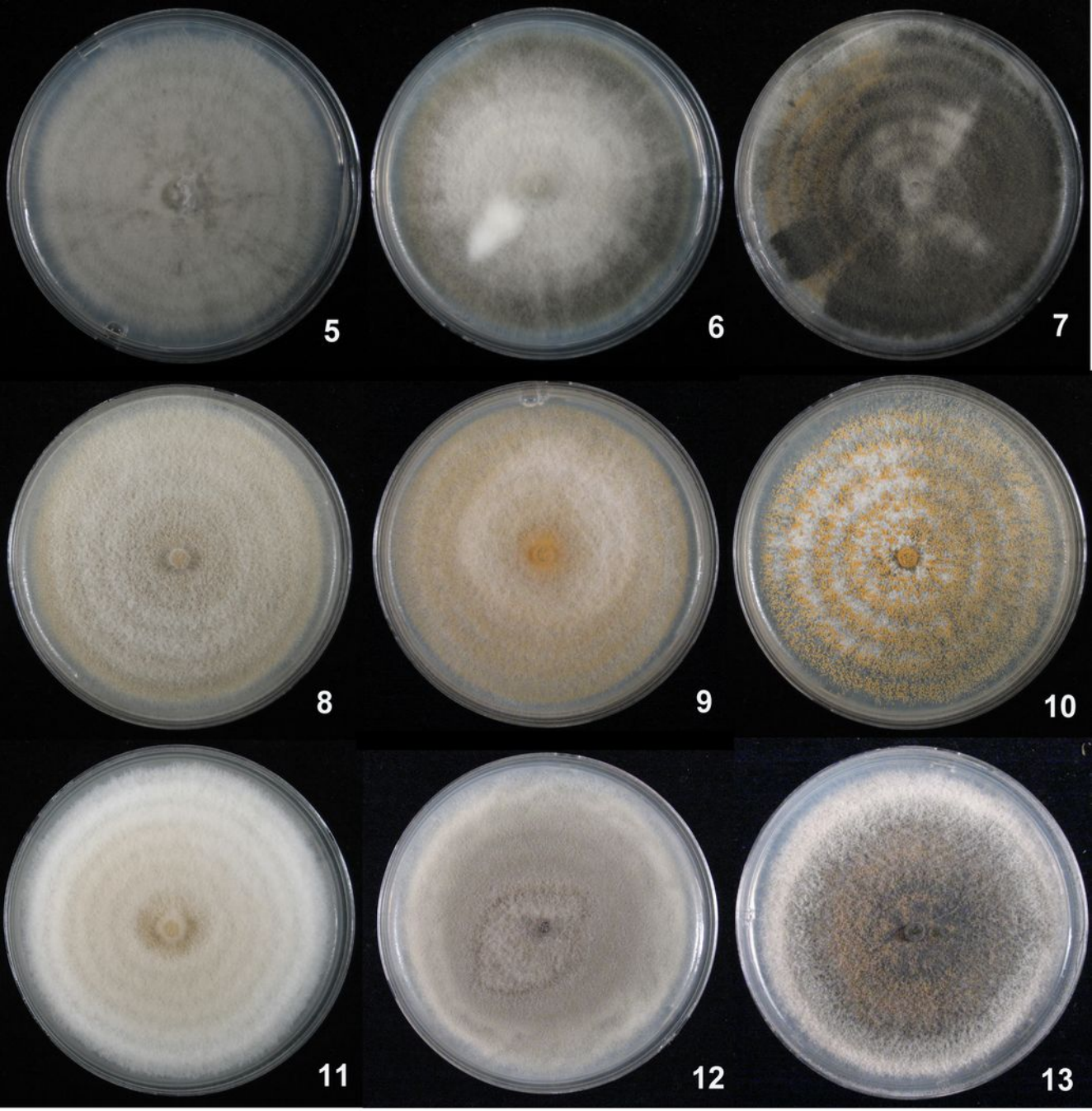
| Character | *C. gloeosporioides* IMI 356878 (A1-1) | *C. tropicale* (A2-1, A3-1) | *C. ignotum* (A4-3, A5-4) | *C. theobromicola* (A7-5) | *Colletotrichum* sp. indet. 1 (A7-5) | *Colletotrichum* sp. indet 2, 3 (A8-7, A9-7)) |
|---|---|---|---|---|---|---|
| Conidium length µm | (10.2) 11.7-14.2(-16.2)<br>12.5–13.4<br>n=30 | (9.5–)12.5–16.5(–21.2)<br>14.1–14.8<br>n=150 | (11.2–)13.5–17.5(–21.2)<br>13.8–14.6<br>n = 180 | (11.5–)14.5–18.7(–23.2)<br>16.2–16.9<br>n= 180 | (12.5–)15.5–20.2(–26.7)<br>17.5–18.2<br>n = 180 | (13–)14.2–17.7(–19.7)<br>15.3–16.6<br>n = 30 |
| Conidium width µm | (4.5-) 4.8-5.8 (-6.5)<br>5.2–5.6<br>n=30 | (4.0–)4.8–5.5(–6.5)<br>5.1–5.2<br>n=150 | (4.0–)4.5–5.7(–6.0)<br>5.0–5.2<br>n = 180 | (3.2–)4.5–5.5 (-7.0)<br>5.0–5.1<br>n=180 | (2.7–)4.2–5.5(–6.0)<br>4.7–4.9<br>n = 180 | (3.7–)4.0–4.5(–4.7)<br>4.2–4.4<br>n = 30 |
| Conidium l/w | (1.7-) 2.1-2.7 (-3.3)<br>2.3–2.6<br>n=30 | (1.2–)2.3–3.3(–4.7)<br>2.7–2.9<br>n=150 | (2.0–)2.5–3.5(–4.0)<br>2.8–3.0<br>n = 180 | (2.4–)2.7–3.7(–3.8)<br>3.3–3.4<br>n=180 | (2.7–)3.0–4.5(–7.0)<br>3.7–3.8<br>n = 180 | (2.9–)3.2–4.4(–4.9)<br>n = 30 |
| Conidium shape | subcylindrical | subcylindrical, rarely clavate | subcylindrical | subcylindrical and clavate | subcylindrical | subcylindrical |
| Phialide length µm | (12.2–)14.5–19.5(–20.5)<br>n = 21 | (7.5–)11.0–16.0(–19.0)<br>n = 120 | (9.2–)16.2–28.7(–42.5)<br>n = 120 | (10.2–)14.2–26.7(–38.0)<br>n=90 | (7.5–)14.0–21.2(–27.0)<br>n = 149 | (12.0–)16.0–23.5(–35.5)<br>n = 30 |
| Phialide widest point µm | (1.7–)2.2–3.2(–3.5)<br>n=21 | (1.7–)2.5–3.5(–4.0)<br>n=120 | (2.2–)3.0–5.2(–8.0)<br>n = 120 | (2.3–)3.0-4.5(–5.7)<br>n=90 | (1.5–)2.2–3.5(–4.7)<br>n = 149 | (2.7–)3.5–5.0(–6.5)<br>n = 30 |
| Phialide proliferation | none observed | percurrent occasional | sympodial occasional | none observed | none observed | percurrent common |
| Swollen cells in the hymenium | – | + | – | – | – | – |
| Setae (+/−) | abundant | rare | rare | absent | absent | absent |
| Perithecia (+/−) | – | – | + | – | – | – |
| ascospores µm | | | (12.0–)13.2–16.7(–19.0)<br>n = 48 | | | |
| Colony diam 96 h, 25 C, on PDA mean plus/minus standard dev | 50<br>n = 1 | 36–56<br>n = 11 | 46–52<br>n = 6 | 46–50<br>n = 3 | 20–24<br>n = 6 | 55<br>n = 1 |
| Colony characters after 10 d at 25 C in light | Uniformly felt-like<br>Concentric rings lacking<br>Abundant conidia | Aerial mycelium scant<br>Concentric rings of conidial production | Aerial mycelium dense, white<br>Colony typically sterile | Aerial mycelium felt-like, dark gray<br>Conidial production sparse | Uniformly felt-like<br>Concentric rings lacking<br>Abundant conidia | Little aerial mycelium<br>Concentric rings obscure<br>Abundant conidia, filling the Petri plate |

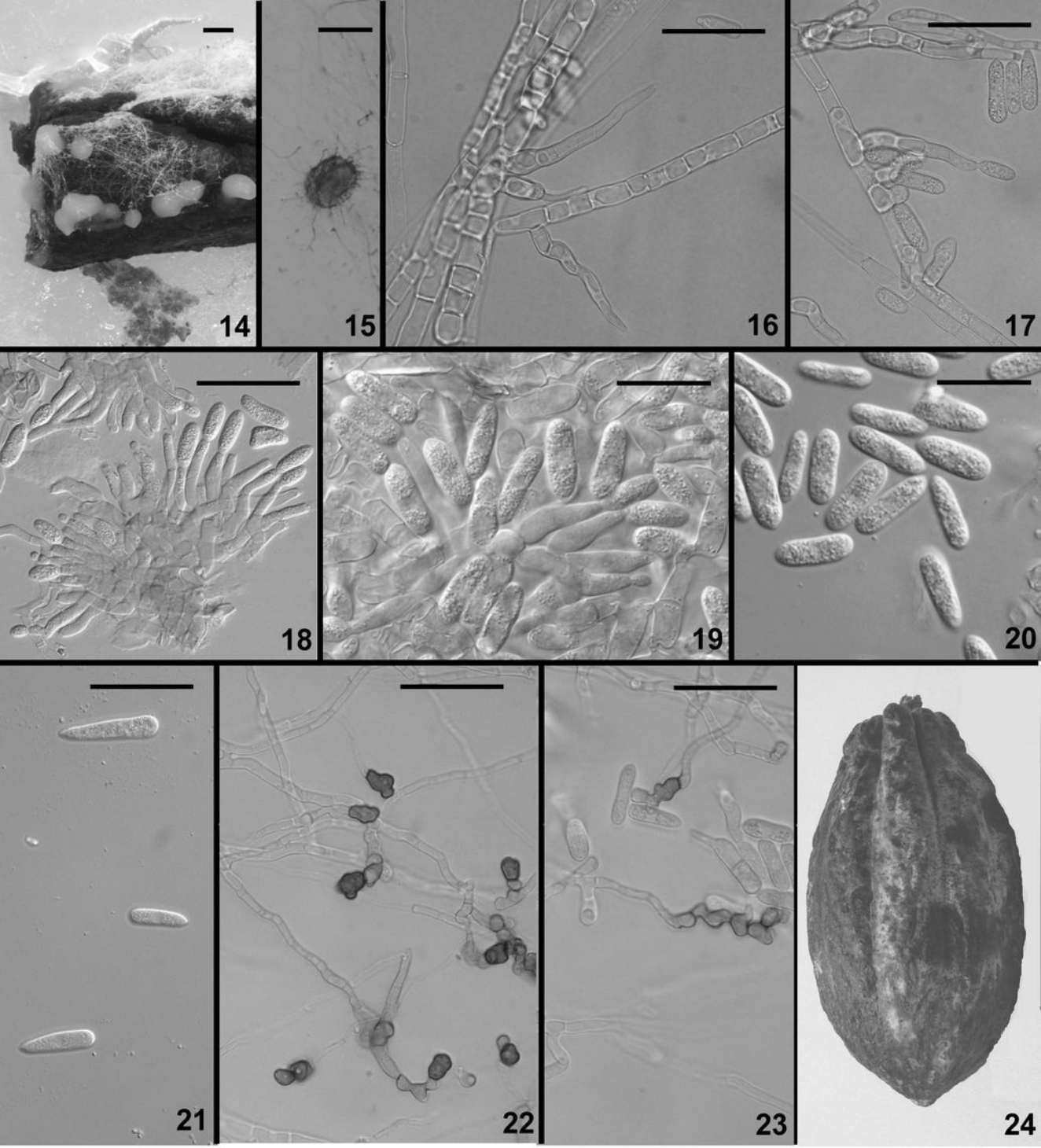

a
8416
Z1093
4226
Z1080
E1456
8453
E406
E704
E347
7585
E2303
E449
E1164
5087
5101
E1153
V1H1
4861
2126
7423
7767
8364
4259
G.J.S.08-42
E649
G.J.S.08-52
5283
E1198
7584
G.J.S.08-56
5806
G.J.S.08-54
8401
Q633
E1533
3870
G.J.S.08-57
G.J.S.08-144
G.J.S.08-145
G.J.S.08-143
G.J.S.08-147
G.J.S.08-146
G.J.S.08-148
E183
1091
1092
8395
E42
E886
E1418
7574
3679
3589
1087
IMI356878
G.J.S.08-51
G.J.S.08-47
G.J.S.08-43
G.J.S.08-46
G.J.S.08-45
G.J.S.08-48
G.J.S.08-50
A
C. gloeosporioides s.l.
4766
1093
E681
4801
E2452
E1249
3386
LCM243
C
Colletotrichum spp. indet.
8975
Z1098
LCM232
Z1090
B
C. boninense
Q855
Q882
E2550
D
Colletotrichum spp. indet.
98
95
100
84
70
100
81
100
1 change
b
8416
Z1093
E1164
Z1080
Q633
5806
4861
E704
E347
E1533
3870
7584
G.J.S.08-54
E449
7585
5087
2126
5101
8453
G.J.S.08-42
8364
7423
5283
E406
G.J.S.08-56
E1153
E649
4226
A2-1
IMI356878
A1-1
E1456
E2303
4259
E1198
8401
A3-2
V1H1
7767
G.J.S.08 52
1091
1092
A8-6
8395
E183
A5-4
G.J.S.08-144
G.J.S.08-147
G.J.S.08-148
G.J.S.08-146
G.J.S.08-145
G.J.S.08-143
A6-4
E42
E886
E1418
7574
3589
1087
3679
A4-3
G.J.S.08 57
A9-7
G.J.S.08-51
G.J.S.08-47
G.J.S.08-46
G.J.S.08-45
G.J.S.08-43
G.J.S.08-48
G.J.S.08-50
A7-5
A
C. gloeosporioides s.l.
8975
Z1098
LCM232
Z1090
B
C. boninense
B1-8
4766
C2-9
1093
E1249
E681
C1-9
LCM243
C3-10
3386
C5-11
4801
E2452
C4-10
C
Colletotrichum spp. indet.
Q855
D3-1
Q882
D2-13
E2550
D1-12
D
Colletotrichum spp. indet.
98
71
92
98
98
99
92
100
100
99
98
84
89
84
76
94
89
5 changes

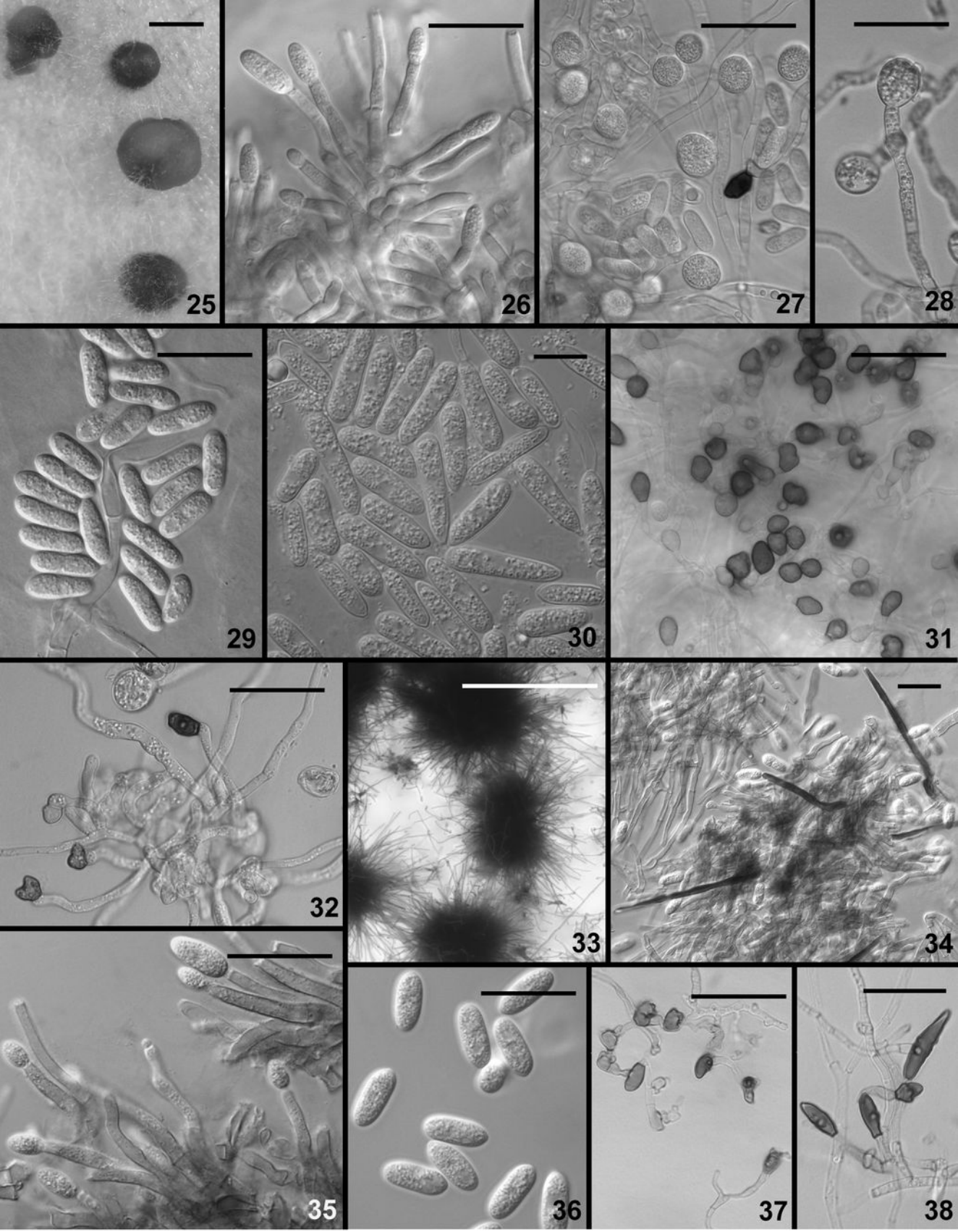

1
2.1
2.2
2.3
3
4.1
4.2
4.3
5
6
GIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYYVTVIDAPGHRDFIKNMITG
Type 1
Type 2
Type 3
Type 4
Type 5
Type 6
Type 7
Type 8
Type 9
Type 10
Type 11
Type 12
Type 13
Type 14
Type 15
A1-1, A2-1, A10-1*, D3-1
A3-2
A4-3, A14-3*
A5-4, A6-4
A7-5
A8-6
A9-7
B1-8
C1-9, C2-9
C3-10, C4-10
C5-11
D1-12
D2-13
A11-14*
A12-15*, A13-15*

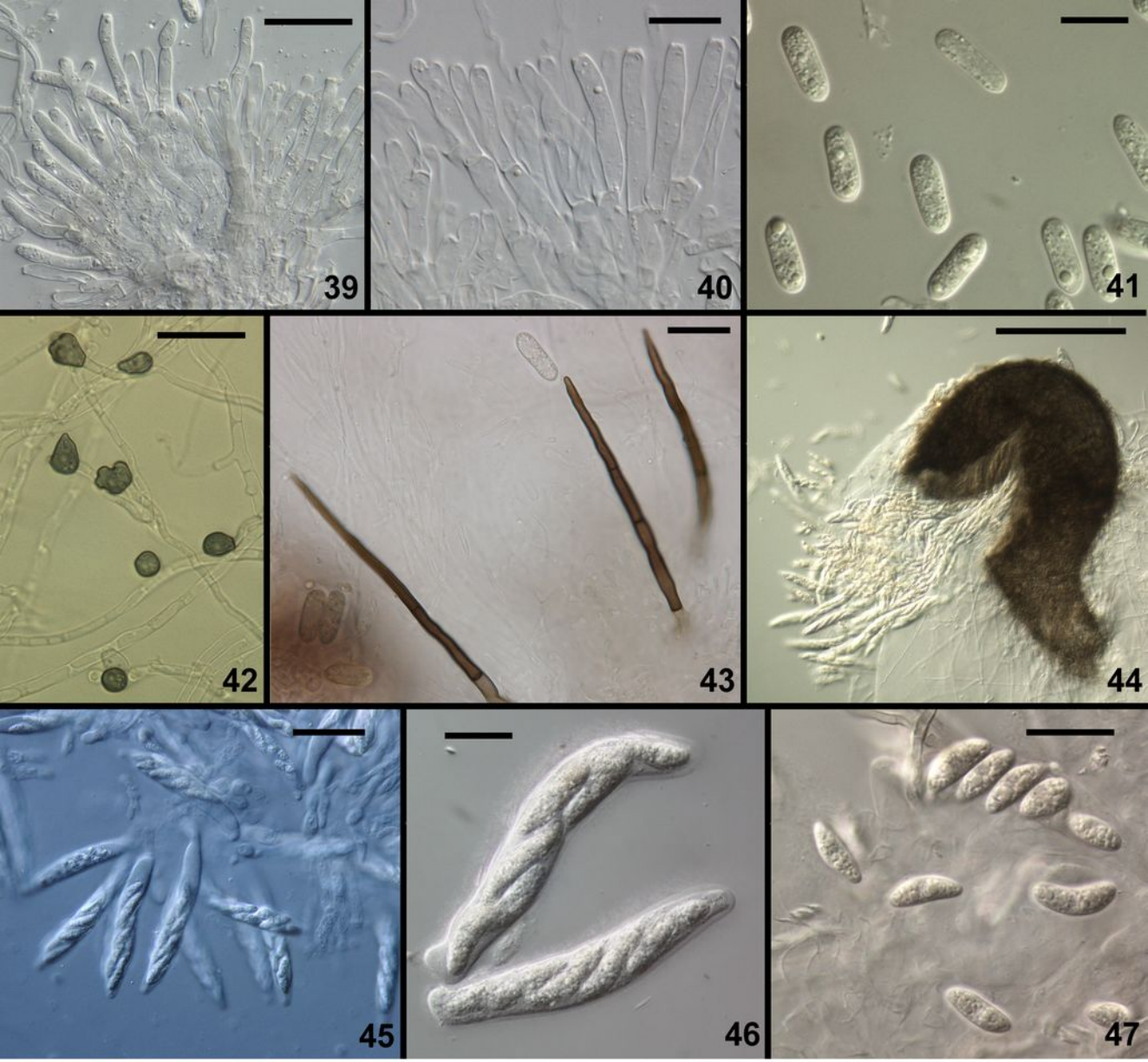

7423
E406
5101
E1164
Q633
E2303
8401
4861
GJS 08-42
C. tropicale
(A2-1, A3-2)
100/100/100
-/97/74
-/96/100
7767
GJS 08-52
1092
100/100/72
94/100/72
100/100/100
Colletotrichum sp.indet. 2
(A8-6)
GJS 08-57
Colletotrichum sp. indet. 3
(A9-7)
GJS 08-144
GJS 08-147
100/84/100
100/100/100
Colletotrichum sp. indet. 1
(A6-4)
1087
3679
3589
E886
7574
E183
8395
99/100/100
100/100/100
100/86/100
100/100/100
C. ignotum
(A4-3, A5-4)
IMI356878
C. gloeosporioides
(A1-1)
GJS 08-48
GJS 08-43
GJS 08-50
100/100/100
C. theobromicola
(A7-5)
100/100/100
100/100/97
4766
4801
3386
C
Colletotrichum sp. indet
0.005 substitutions/site

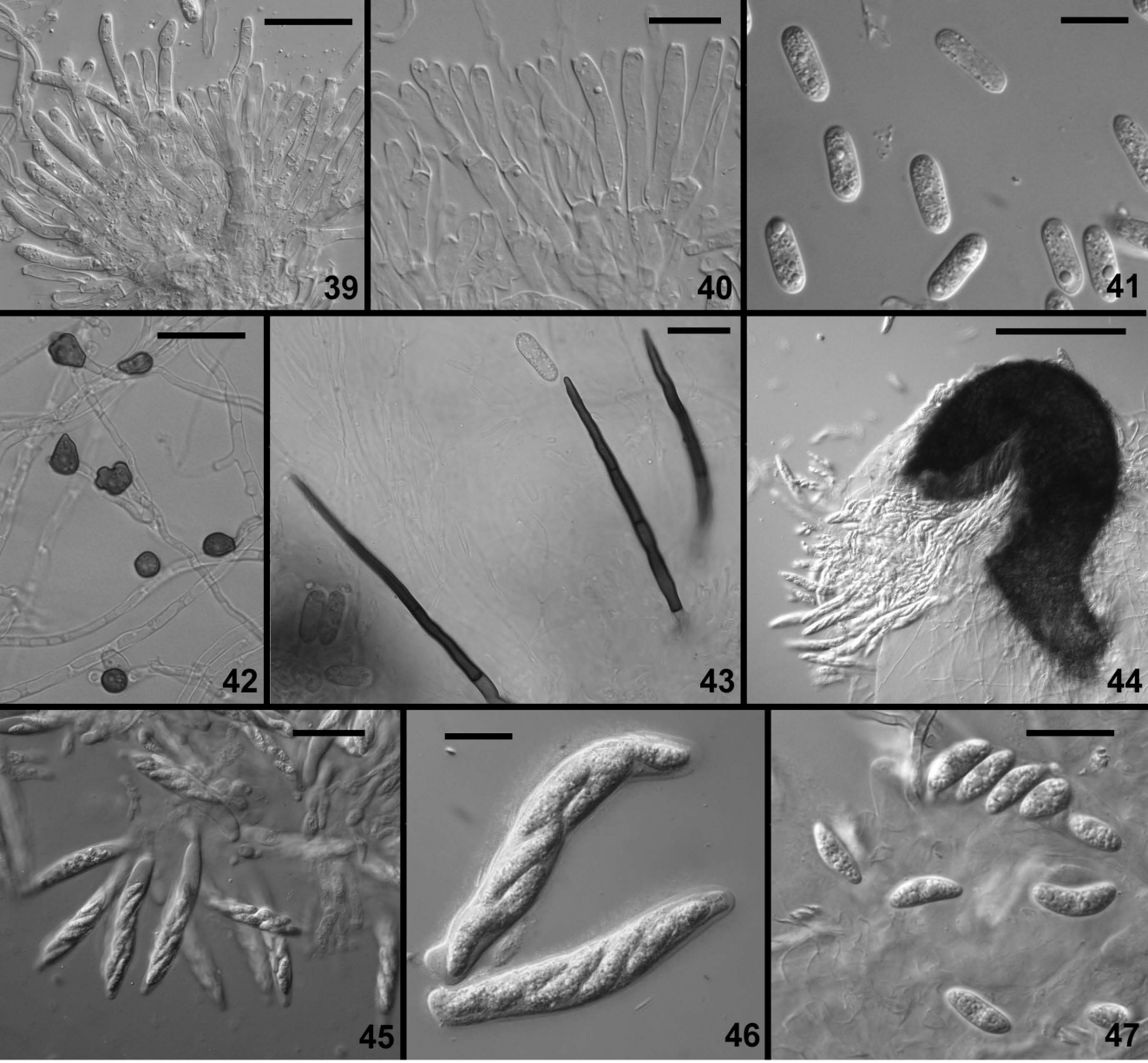

100
E1164
Q633
C. tropicale A3-1
A2-107
B1-122
B3-131
C2-154
C1-152
C3-155
C3-148
A2-108
C3-161
C3-147
100
A10-1*
100
C3-156
A12-15*
88
A2-102
B2-124
B2-126
C2-143
100
A13-16*
A2-105
B2-165
B2-169
A3-173
C3-160
C2-138
B2-125
B2-167
93
A14-3*
A3-176
1091
7767
95
Colletotrichum sp. indet. 2
A8-6
B2-127
C1-134
C1-132
C2-136
94
A11-14*
GJS08-47
GJS08-51
C. theobromicola
A7-5
5 changes


5
6
7
8
9
10
11
12
13


14
15
16
17
18
19
20
21
22
23
24


25
26
27
28
29
30
31
32
33
34
35
36
37
38


39
40
41
42
43
44
45
46
47


39
40
41
42
43
44
45
46
47