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Espécies de *Colletotrichum* associadas à antracnose da banana no Brasil e sensibilidade de *C. musae* ao tiofanato-metílico

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Tese apresentada ao Programa de Pós-Graduação em Fitopatologia da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Fitopatologia.

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"I've made mistakes but I believe
That everything was worth the fight
Cause in the end, the road is long
But only cause it makes you strong
It's filled with peaks and twists and turns
Sometimes you have to learn forget about it"

Marina and the Diamonds

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"Ainda que eu andasse pelo vale da sombra da morte, não temeria mal algum, porque tu estás comigo; a tua vara e o teu cajado me consolam." (Sl. 23:4)

Ao grande DEUS,

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SUMÁRIO

RESUMO GERAL

Espécies de *Colletotrichum* são comumente associadas com a antracnose de uma ampla gama de plantas hospedeiras, incluindo fruteiras tropicais cultivadas. A antracnose é a doença pós-colheita mais séria da banana e está amplamente distribuída em todas as regiões produtoras. Embora o Brasil seja um dos maiores produtores mundiais de banana, a diversidade de espécies de *Colletotrichum* associadas à antracnose da banana no Brasil era desconhecida antes do presente estudo. As espécies *C. musae*, *C. tropicale*, *C. dianesei* *C. fragariae*, e uma nova espécie descrita como *C. chrysophilum* são aqui relatadas em associação com a antracnose da banana. Todas as espécies foram patogênicas em frutos de bananeira, mas variaram de acordo com a virulência. Isolados da espécie *C. musae*, a qual é prevalente nos cultivos de banana no Brasil, foram avaliados quanto a sensibilidade de *C. musae* ao tiofanato-metílico, um dos fungicidas mais utilizados em plantios de banana no Brasil. De acordo com o teste de sensibilidade in vitro ao tiofanato-metílico, foi detectada a presença de isolados moderadamente resistentes cuja concentração do fungicida na qual o desenvolvimento do fungo foi inibido em 50% variou de 10.43 to 48.73 μg/ml. Os isolados moderadamente resistentes apresentaram uma substituição de TAC para TTC no códon 200 do gene β-tubulina. A dose comercial do tiofanato-metílico apresentou baixa eficácia no controle de isolados moderadamente resistentes em frutos de banana. Não foram observadas diferenças estatísticas entre isolados sensíveis e moderadamente resistentes quando aos componentes de fitness avaliados ($P>0.05$), indicando que a ausência de custo de fitness para os isolados moderadamente resistentes.

GENERAL ABSTRACT

Species of *Colletotrichum* are commonly associated with anthracnose of a wide range of host plants, including cultivated tropical fruits. Anthracnose is the most serious post-harvest disease of banana and is widely distributed among the banana production regions of the world. Although Brazil is one of the largest producers of banana worldwide, the diversity of *Colletotrichum* species associated with banana anthracnose in Brazil was unknown before the present study. Here we report *C. musae*, *C. tropicale*, *C. dianesei*, *C. fragariae* and a new species described as *C. chrysophilum* in association with banana anthracnose in Brazil. All species were pathogenic on banana fruit, but vary with respect to virulence. For some isolates of C. musae, the prevalent species in all banana growing areas, was assessed the sensitivity to the thiophanate-methyl, one of the fungicides most used in banana orchards in Brazil. Based on the thiophanate-methyl sensitivity in vitro test, was detected the presence of isolates moderately resistant with the concentration of the fungicide at which fungal development is inhibited by 50% ranged from 10.43 to 48.73 μg/ml. Moderately resistant isolates presented a substitution of TAC for TTC at codon 200 in β-tubulin gene. Thiophanate-methyl formulation at the label rate showed low efficacy in controlling the moderately resistant isolates in banana fruits. No statistical differences were observed between the sensitive and moderately resistant isolates for the fitness components evaluated ($P>0.05$), indicating the absence of fitness cost for the moderately resistant isolates.

Capítulo I

Introdução

INTRODUÇÃO GERAL

1. A cultura da banana

A bananeira (*Musa* spp.), com origem no Continente Asiático, é encontrada principalmente nas regiões tropicais em todo o mundo, estando presente em mais de 80 países, ocupando área superior a 4 milhões de hectares e produção que ultrapassa de 95 milhões de toneladas. Existem cerca de 180 variedades de bananas, sendo que no Brasil são conhecidas cerca de 35 variedades distribuídas em bananeiras ornamentais, industriais e comestíveis (FAO, 2013). O centro de origem da maior parte do germoplasma de banana está localizado na Ásia, porém ocorrem centros secundários na África Oriental, em algumas ilhas do Pacífico e uma considerável diversidade genética na África Ocidental regiões com clima tropical quente e úmido (SHEPHERD, 1984).

A bananeira é um vegetal herbáceo completo, apresentando caule (rizoma), raiz, folhas, flores, frutos e sementes, e é classificada como uma cultura perene, uma vez que novos perfilhos nascem da base da planta-mãe (BORGES; SOUZA; ALVES, 2000).

Para a produção das cultivares de bananas comestíveis, participaram as espécies selvagens diplóides representadas pelas letras A (*M. acuminata*) e B (*M. balbisiana*). Cada cultivar contém combinações variadas de genomas dessas espécies parentais, cujas combinações resultam os grupos diplóides (AA, BB e AB), triplóides (AAA, AAB e ABB) e tetraplóides (AAAA, AAAB, AABB e ABBB) (COSTA, 2008). As cultivares mais difundidas no Brasil são: Prata, Pacovan, Prata Anã, Maçã, Mysore, Terra e D'Angola, do grupo AAB, utilizadas unicamente para o mercado interno, e Nanica, Nanicão e Grande Naine, do grupo AAA, usadas principalmente no mercado para exportação. Em menor escala são plantadas 'Ouro' (AA), a 'Figo Cinza' e 'Figo Vermelho' (ABB), 'Caru Verde' e 'Caru Roxa' (AAA). As cultivares Prata, Prata Anã e Pacovan são responsáveis por aproximadamente 60% da área cultivada com banana no Brasil (BORGES; SOUZA; ALVES, 2000).

A temperatura ótima para o desenvolvimento normal das bananeiras comerciais situa-se em torno dos 28°C. Considera-se a faixa de 15°C a 35°C de temperatura como os limites extremos para a exploração racional da cultura. Havendo suprimento de água e de nutrientes, essa faixa de temperatura induz ao crescimento máximo da planta. Quanto à precipitação, as maiores produções de banana estão associadas a uma precipitação total anual de 1.900 mm,

bem distribuída no decorrer do ano, ou seja, representando 160 mm/mês e 5 mm/dia, e por ser uma planta típica das regiões tropicais úmidas, apresenta melhor desenvolvimento em locais com médias anuais de umidade relativa superiores a 80% (BORGES; SOUZA, 2004).

A produção mundial de banana se concentra em alguns países como: Índia, China, Filipinas, Brasil, Equador e Indonésia, correspondendo a 64,3% da produção mundial. O Brasil se destaca como o quinto maior produtor mundial, produzindo no ano de 2011 cerca de 6,8 milhões de toneladas com área cultivada de mais de 479 mil hectares (FAO, 2013).

A bananicultura representa um importante papel econômico e social, movimentando cerca de 2 bilhões de dólares ao ano. Dentre todos os produtos agrícolas produzidos no país no ano de 2009 a banana foi a 10ª *commodities* em termos de quantidade produzida, no entanto foi exportada somente 2,2% da produção total, o equivalente a 143,9 mil toneladas (FAO, 2013).

Segundo dados do IBGE, no ano de 2010 a cultura da banana ocupou uma área plantada de 483.562 hectares e uma produção de 6.783.482 toneladas. Seu cultivo é realizado em todos os Estados, sendo a região Nordeste a maior produtora, seguida das regiões Sudeste, Sul, Norte e Centro-Oeste. Isoladamente, o estado de São Paulo encontra-se como o maior produtor da fruta no Brasil, com 1.257.539 toneladas, o equivalente a 19% da produção nacional (IBGE, 2010).

Dentre os fatores limitantes desta cultura, incluem-se os danos físicos (injúrias mecânicas), desordens ou distúrbios fisiológicos, e os danos causados decorrente das infecções por diversos microrganismos, fatores estes que também estão aliados ao baixo nível tecnológico adotado nos pomares (MAIA et al., 2008; SILVA et al., 2003; RANGEL; PENTEADO; TONET, 2002; CARDOSO, 2005).

Inúmeros microrganismos como fungos, bactérias, nematoides e vírus, prejudicam o desenvolvimento da bananeira ao afetar diversas partes da planta. Dentre esses microrganismos, merece destaque na pós-colheita desta cultura o fungo mitósporico *Colletotrichum* spp., agente etiológico da antracnose da banana (SILVA; CORDEIRO, 2000; CORDEIRO; MATOS; KIMATI, 2005; CORDEIRO; MATOS, 2005; PESSOA et al., 2007).

2. A antracnose da banana

A antracnose representa o mais grave problema na pós-colheita de banana e está amplamente distribuída em todas as regiões produtoras desta fruta no mundo (WARDLAW, 1972; CORDEIRO; MATOS, 2000).

O agente etiológico geralmente infecta os frutos verdes no campo (pré-colheita), permanecendo quiescente até o início da maturação; em pós-colheita, a infecção quiescente manifesta-se durante o período de transporte e maturação dos frutos e ocasionam outras infecções, caracterizando a fase de infecção não-quiescente. Normalmente, nenhuma lesão desenvolve-se em frutos verdes no campo. A doença caracteriza-se pela formação de lesões escuras e deprimidas sobre as quais em condições de alta umidade, aparecem frutificações mucilaginosas do fungo. Com o progresso da doença, as lesões aumentam de tamanho, podendo coalescer, formando grandes áreas necróticas (CORDEIRO; MATOS, 2000; CORDEIRO; MATOS; KIMATI, 2005; CORDEIRO; MATOS, 2005), afetando consideravelmente sua qualidade e comprometendo a comercialização (ABAYASEKARA; RATNAYAKE; ADIKARAM, 1998; SPONHOLZ et al., 2004).

Pequenas manchas marrons começam a aparecer em todas as partes da casca de frutos em estádio de amadurecimento. Estas manchas se expandem e coalescem para formar grandes lesões de coloração marrom a preto, deprimidas, que muitas vezes são cobertos por massas de conídios dos fungos, as quais são identificadas como pontuações de coloração laranja ou salmão. A polpa somente é afetada após frutos atingirem avançado estado de maturação. As lesões que se desenvolvem a partir de injúrias em frutos verdes são de coloração preta, acompanhando as dimensões e profundidade da injúria. Estas lesões podem ser delimitadas por halos cloróticos e podem invadir a celulose sob condições de temperatura elevada (PLOETZ; THOMAS; SLAGAUGH, 2003).

Colletorichum é um componente comum da microflora no interior da cobertura da bananeira. Trabalhos realizados em Guadalupe indicaram que as peças florais e brácteas são as mais importantes fontes de inoculo em campo, e que uma tripla redução na severidade da doença foi realizado quando estas fontes foram removidas durante a floração (DE LAPEYRE DE BELLAIRE et al., 2000). Os conídios são formados sob condições de elevada umidade e são dispersos pela chuva, primariamente dentro da mesma planta e posteriormente entre plantas. Chuva também é necessário para a infecção. Em Guadalupe, a gravidade da doença foi significativamente correlacionada com precipitação acumulada durante os primeiros 35 dias após o florescimento, e a doença foi completamente controlada quando os cachos foram protegidos da chuva com capas. *Colletotrichum* forma apressórios melanizados na casca de fruta logo após a germinação dos conídios. Estas infecções normalmente permanecem latentes até que a maturação seja iniciada (PLOETZ; THOMAS; SLAGAUGH, 2003).

Quanto às espécies de *Colletotrichum* ascociadas à antracnose em banana, Su et al. (2011) epitipificaram a espécie *Colletotrichum musae* (Berk. & M.A. Curtis) Arx através de

características fenotípicas e moleculares, associando esta espécie ao isolado tipo oriundo de bananas da América do Norte, e a isolados obtidos de bananas em províncias do norte da Tailândia. Em 2013 foi primeiramente relatada a espécie *Colletotrichum gloeosporioides* (Penz.) Penz. et Sacc. causando antracnose em bananas na Malásia (INTAN SAKINAH; SUZIANTI; LATIFFAH, 2013). Em um trabalho de investigação da etiologia das espécies causadores de antracnose de fruteiras tropicais, um isolado causador de antracnose em banana, oriundo da Tailândia, foi associado ao complexo de espécies *C. siamense sensu lato* (UDAYANGA et al., 2013).

3. O gênero *Colletotrichum*

Os fungos do gênero *Colletotrichum* e seu teleomorfo *Glomerella* encontram-se distribuídos por todo o mundo, especialmente como causadores de problemas na pré e na pós-colheita nas regiões tropicais (SUTTON, 1992).

A circunscrição de espécies para o gênero *Colletotrichum* foi por longa data realizada com base em critérios descritivos clássicos, como dimensões dos conídios, presença, ausência e morfologia das setas. Outros critérios complementares foram incluídos posteriormente, como a gama de hospedeiros e os tipo de dano causado (SUTTON, 1980). Cerca de 900 espécies já foram descritas ou transferidas para o gênero *Colletotrichum* (BAILEY; JEGER, 1992). A circunscrição de espécies para o gênero *Colletotrichum* foi por longa data realizada com base em critérios descritivos clássicos, como dimensões dos conídios, presença, ausência e morfologia das setas. Outros critérios complementares foram incluídos posteriormente, como a gama de hospedeiros e os tipo de dano causado (SUTTON, 1980).

Segundo Von Arx. (1957), Stoneman, em 1898, encontrou a forma ascógena em antracnose provocada por espécies de *Gloeosporium*, e as colocou em um novo gênero então denominado *Gnomoniopsis*. No entanto, esse nome já havia sido ocupado com outro fungo por Berlese em 1892. Em 1903, Von Schrenk e Spaulding mudaram a classificação para *Glomerella*, contendo cinco espécies. Em 1957, Von Arx identifica que o conídio da espécie tipo do gênero tem duas células, o que foi aceito posteriormente, sendo transferido para o gênero *Marsonina*. Ainda neste mesmo ano, Von Arx concluiu que vários anamorfos dos gêneros *Colletotrichum*, *Vermicularia* e *Gloeosporium* pertenciam ao mesmo grupo, e aceitou o nome *Colletotrichum* proposto por Corda, e o número de espécies foi drasticamente reduzido de várias centenas para 11. Posteriormente, Sutton critica a classificação de Von Arx, afirmando que esta foi baseada em informações experimentais insuficientes, além de

uma inadequada examinação ou citação do material original, e em 1980 publica uma compilação de opiniões contemporâneas relacionadas à taxonomia de Coelomycetes, onde o gênero *Colletotrichum* foi dividido em 22 espécies, e espécies como *C. gloeosporioides*, *C. dematium*, *C. capsisi* (Sydow) E. Butler & Bisby e *C. sublineolum* P. Henn. Kabat & Bubák foram designados como grupos de espécies. Esse número de espécies continuou variável com o passar dos anos, variando de 40 em Sutton (1992) a 60 no Dicionário de Fungos (KIRK et al., 2008).

A identificação de espécies de *Colletotrichum* baseada na morfologia tem sido sempre problemática, pois são poucas as características confiáveis, e muitas dessas características são plásticas, variando conforme os métodos e condições experimentais (CAI et al., 2009). A diferenciação baseada no hospedeiro ou gama de hospedeiros não é consistente, pois *taxa* como *C. acutatum*, *C. gloeosporioides* e *C. graminicola* (Ces.) Wils, dentre outros, infectam uma ampla gama de hospedeiros. Outro aspecto é que pelo conceito de espécie biológica, grupos de isolados patogênicos e não patogênicos, podem ambos ser incluídos em uma única espécie (SUTTON, 1980, 1992).

Devido à taxonomia convencional do gênero *Colletotrichum* não ser satisfatória, há a tendência de se utilizar um estudo polifásico para a identificação, o que reflete na classificação de espécies e taxa sub-específicos dentro do gênero (SUTTON, 1992; CANNON; BRIDGE; MONTE, 2000). O que se sugere é que uma abordagem polifásica deva ser adotada antes de novas espécies de *Colletotrichum* serem introduzidas ou epitipos serem designados, devendo-se incorporar dados moleculares, morfológicos, fisiológicos e relacionados à patogenicidade (CAI et al., 2009).

A utilização de caracteres moleculares como dados de sequências de DNA apresenta-se como uma ferramenta vantajosa para a taxonomia de fungos, uma vez que podem ser analisadas estatisticamente para inferir relações filogenéticas. A combinação dos conceitos de espécie filogenética com caracteres moleculares pode superar a maioria das limitações associadas ao conceito biológico e outros conceitos de espécie (SHENOY; JEEWON; HYDE, 2007). Com o rápido progresso dos métodos de filogenia molecular, atualmente é possível reconhecer clados estáveis e bem resolvidos dentro do gênero *Colletotrichum* (CAI et al, 2009).

A região ITS (Internal Transcribed Spacer) do 5.8S rDNA é a mais amplamente sequenciada em estudos filogenéticos do gênero *Colletotrichum*, e consequentemente, primers baseados nessa região (MILLS et al., 1992) foram utilizados nos últimos 10-15 anos como um primer espécie-específico para *C. gloeosporioides*. No entanto, atualmente já são conhecidas

as limitações do ITS e outros marcadores, e o desenvolvimento e uso de *loci* mais informativos tornou-se cada vez mais necessário (SILVA et al., 2012).

Cai et al. (2009) sequenciaram os loci ITS, GPDH (glicerol-3-fosfato desidrogenase) CAL (calmodulina), ACT (actina), CHS (quitina sintase), e EF1α (fator de alongação 1α) de 64 isolados de *C. gloeosporioides senso lato*, distribuídos em 5 grupos através de filogenia multigênica e os avaliaram quanto à separabilidade e heterogeneidade. Quanto à separabilidade, que é a capacidade do gene em separar os grupos (variabilidade inter-específica), os genes GPDH, CAL e ACT apresentaram os maiores valores, sendo considerados bons candidatos para identificação de espécies. Quanto à heterogeneidade (variação intra-específica), conforme esperado, os valores foram mais baixos que os de separabilidade, com exceção do gene EF1α, que apresentou valores maiores devido à alta variabilidade de íntrons, sendo um gene bastante informativo para estudos em nível de população. Em trabalho similar com espécies de *Colletotrichum* de conídio falcado em hospedeiros herbáceos, dentre os genes ITS, ACT, CHS, GPDH, histona 3 e β-tubulina, a separabilidade de espécies foi maior para os genes GPDH e histona 3 (DAMM et al., 2009).

Doyle et al. (2013) analisaram individualmente 4 loci (ITS, TUB2, Apn2 e Apn2/MAT IGS) em estudo filogenético envolvendo isolados do complexo de espécies *C. gloeosporioides*. Foi observado que a região ITS não fornece resolução adequada nem para a atribuição de espécies, nem para avaliar de forma confiável as relações filogenéticas dentro do complexo de espécies C. gloeosporioides. As demais regiões analisadas apresentaram uma melhor resolução filogenética quando comparados à região ITS, sende revelados um maior número de clados terminais. No entanto, a região Apn2/MAT IGS apresentou uma melhor resolução filogenética que os demais genes analisados. Sharma et al. (2013) também comprovaram o poder de resolução filogenética da região Apn2/MAT IGS, sendo identificadas diversas linhagens dentro do clado previamente denominado *C. siamense*, o qual é considerado atualmente como o complexo de espécies *C. siamense*. As espécies inseridas neste complexo parecem ter divergido recentemente, apresentando baixa distinção filogenética representada por clados fracamente suportados para a maioria dos loci comumente utilizados nas análises filogenéticas (UDAYANGA et al., 2013).

4. O controle da antracnose da banana

O controle da antracnose da banana deve começar no campo, com boas práticas culturais, ainda na pré-colheita. Na fase de colheita e pós-colheita todos cuidados devem ser

tomados de forma a evitar ferimentos nos frutos, que são a principal via de penetração dos patógenos. As práticas de despencamento, lavagem e embalagem devem ser executadas com manuseio extremamente cuidadoso dos frutos e medidas rigorosas de assepsia (PLOETZ; THOMAS; SLABAUGH, 2003).

Outras formas de se controlar a doença é através da redução na temperatura de armazenamento (SILVA et al., 2008) e o uso de fungicidas químicos, sendo os liberados para uso nesta cultura em pós colheita, pertencentes ao grupo dos benzimidazóis (AGROFIT, 2010).

Pulverizações ou imersão de frutos na pós-colheita, em fungicidas, especialmente benzimidazóis como o carbendazim, são comumente usados para o controle da antracnose. Entretanto, o surgimento de populações de isolados resistentes do patógeno tem reduzido cada vez mais a efetividade destes produtos (SPONHOLZ et al., 2004). Embora o controle químico da antracnose da banana seja realizado na pós-colheita, o surgimento se populações de isolados de *Colletotrichum* resistentes aos benzimidazóis pode estar associado à utilização de fungicidas deste mesmo grupo químico, como o benomyl, carbendazim, tiofanato metilico e thiabendazole, os quais são utilizados em campo para o controle de outras doenças da cultura (FANCELLI, 2003).

5. Resistência de fungos a fungicidas

Os benzimidazóis atuam principalmente através da ligação à tubulina dos fungos, interferindo na mitose e na formação do citoesqueleto dos fungos. O mecanismo de resistência aos fungicidas benzimidazóis tem sido examinado em diferentes fungos fitopatogênicos (DAVIDSE, 1986).

Resistência a fungicidas benzimidazóis tem sido detectada em várias espécies de fungos. Na maioria dos casos, a resistência foi correlacionada com mutações pontuais no gene da β-tubulina, o que resulta em sequências de aminoácidos alterados no sítio de ligação dos benzimidazóis. Os resultados de vários estudos demonstraram que alterações nos códons 6, 50, 167, 198, 200, e 240 no gene da β-tubulina podem causar resistência a benzimidazóis em isolados de fungos fitopatogênicos (MA; MICHAILIDES, 2005). O envolvimento direto das mutações conferindo resistência a benzimidazóis tem sido comprovado através de ensaios utilizando mutagênese induzida nestes sítios, seguido da recomposição do gene para a forma não mutagênica (LI et al., 1996).

Em certos agentes patogênicos, é evidente que as propriedades genéticas como diploidia durante a maior parte do ciclo de vida, ou a presença de introns adjacentes ao sítio alvo das mutações, podem suprimir a expressão da resistência. O risco de resistência é intensificado se os mutantes resistentes têm adaptabilidade normal, no que diz respeito ao crescimento, reprodução e patogenicidade (BRENT; HOLLOMON, 2007).

Testes de adaptabilidade em laboratório devem envolver ensaios como taxa de crescimento e grau de esporulação in vitro e patogenicidade em plantas hospedeiras. Falhas ou reduções graves nestas variáveis, dos isolados resistentes sugerem que essa resistência pode não resultar em problemas práticos. Experimentos de competição, utilizando isolados sensíveis e resistentes, também podem indicar diferenças de adaptabilidade. Se os resistentes são normais ou melhores do que o normal, no seu crescimento, patogenicidade e esporulação, isso indica um sinal de risco (BRENT; HOLLOMON, 2007).

Com base nos pontos supracitados e diante da importância desta doença para a cultura da banana na pós-colheita, é de grande importância o conhecimento sobre a identidade das espécies que ocorrem no Brasil, através de estudos baseados em características fenotípicas e moleculares do agente causal, uma vez que as estratégias de manejo podem diferir conforme a espécie prevalente, bem como da diversidade destes isolados quanto à resistência aos principais fungicidas utilizados na cultura e os mecanismos moleculares envolvidos nessa resistência.

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Capítulo II

Exploring data quality in *Colletotrichum* systematics with a case study on *Musa*-associated species

Exploring data quality in *Colletotrichum* systematics with a case study on *Musa*-associated species.

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RESUMO

Várias espécies de *Colletotrichum* são comumente associadas com a antracnose de fruteiras tropicais cultivadas. A antracnose é a doença pós-colheita mais séria da banana e está amplamente distribuída mundialmente em todas as regiões produtoras. Embora o brasil seja um dos maiores produtores mundiais de banana, a diversidade de espécies de *Colletotrichum* associadas à antracnose da banana no Brasil era desconhecida antes do presente estudo. Aqui são relatadas as espécies *C. musae*, *C. tropicale* e *C. dianesei* em associação com a antracnose da banana. Nós também encontramos um único isolado identificado como *C. fragariae*, que é reintroduzida como espécie válida. Quatro linhagens não se agruparam com nenhuma espécies previamente descrita, e são consideradas novas linhagens. Três linhagens permaneces sem descrição, e uma é descrita como *C. chrysophilum* sp. nov. Todas as espécies foram patogênicas em frutos de bananeira, mas variaram de acordo com a virulência. A partir

de uma perspectiva metodológica, nós encontramos que a escolha do algoritmo de filtragem de alinhamento (GBLOKS × Guidance2) pode ter um impacto significativo sobre a inferência da topologia, e que o Guidance2 retém mais sítios filogeneticamente informativos. Da mesma forma, a escolha do marcador pode ter um impacto na definição das espécies se a falta de sinal filogenético é confundida com uma forte discordância. Os loci mais informativos, de acordo com perfis de informatividade filogenética, são APN2, APN2/MAT-IGS, CAL, GAPDH, GS, TUB2, e o novo marcador, GAP2-IGS. Finalmente, descobrimos que a variação na morfologia de conídios induzida por diferenças em meios de cultura pode levar à incapacidade de se diferenciar espécies que, de outro modo, podem ser distinguidos com base das diferenças fenotípicas.

ABSTRACT

Several *Colletotrichum* species are commonly associated with anthracnose of tropical fruits. Anthracnose is the most serious post-harvest disease of banana and is widely distributed among the banana production regions of the world. Although Brazil is one of the largest producers of banana worldwide, the diversity of *Colletotrichum* species associated with banana anthracnose in Brazil was unknown before the present study. Here we report *C. musae*, *C. tropicale*, and *C. dianesei* in association with banana anthracnose. We also found a single isolate identified as *C. fragariae*, which is reintroduced as a valid species. Four lineages did not cluster with any previously described species, one of which is described as *C. chrysophilum sp. nov*. All species are pathogenic on banana fruit, but vary with respect to virulence. From a methodological perspective, we find that the choice of alignment filtering algorithm, (GBLOCKS vs. Guidance2) can have a significant impact on topological inference and that Guidance retains more phylogenetically informative sites. Similarly, the choice of marker can have an impact on species delimitation if lack of phylogenetic signal is

confounded with strong discordance. The most informative loci, according to phylogenetic informativeness profiling, are APN2, APN2/MAT-IGS, CAL, GAPDH, GS, TUB2, and a new marker, GAP2-IGS. Finally, we find that variation in conidial morphology induced by differences in culture media can lead to the inability to differentiate species that may otherwise be distinguishable on the basis of phenotypic differences.

Key words: alignment, anthracnose, culture media, gene tree discordance, phylogenetic informativeness, multilocus, species delimitation.

Taxonomic novelties: New species – *C. chrysophilum* W.A.S. Vieira, W.G. Lima, M.P.S. Câmara & V.P. Doyle. **Ressurected names –** *C. fragariae* A.N. Brooks.

INTRODUCTION

Colletotrichum Corda is an important and widespread fungal genus with species that are serious plant pathogens or frequent endophytes in aerial organs (Rodrigues & Petrini 1997, Suryanarayanan *et al.* 2002, Cannon & Simmons 2002, Hyde *et al.* 2009). As plant pathogens, *Colletotrichum* spp. are the principal cause of anthracnose and postharvest fruit rots, as well as the causal agents of many damping-off and blossom and seedling blight diseases (Bailey & Jeger 1992). While anthracnose can affect the production of high-value fruits, such as strawberry, mango, citrus and avocado, it also causes significant yield loss in staple crops such as banana (Cannon *et al.* 2012).

The etiology of banana anthracnose, which mainly occurs on fruit (Simmonds & Mitchell, 1940) and starts as a quiescent infection on the immature epicarp (Lim *et al.* 2002), has long been associated with *C. musae*. *Colletotrichum musae* was originally described from North Carolina (Berkeley 1874) and recently epitypified by Su *et al.* (2011). However, other

79 species have also been found in association with rotting banana fruit, including *C. karstii* (*C.*
80 *boninense* species complex - Damm *et al.* 2012a), *C. paxtonii* (*C. acutatum* species complex -
81 Sherriff *et al.* 1994, Johnston & Jones 1997, Damm *et al.* 2012b), *C. gloeosporioides sensu*
82 *stricto* (Intan Sakinah *et al.* 2013), and one undescribed species from *C. siamense sensu lato*
83 (Udayanga *et al.* 2013), the latter two of which are in the *C. gloeosporioides* species complex.
84 However, these reports of *Colletotrichum* species associated with banana anthracnose are
85 restricted to the United States, Thailand, Malaysia, Mexico, Santa Lucia and the West Indies,
86 none of which are among the top five producers of banana on the global market

87 Brazil is the fifth-largest producer of banana (*Musa* spp. L.) worldwide, constituting
88 about 6.8% (7 M tons) of overall global production annually, about 96 000 tons of which are
89 exported (FAO 2015). However, *Colletotrichum*, among other post-harvest pathogens,
90 impacts the quality and limits the exportability of Brazilian fruit, preventing long-distance
91 transport and entry into international markets (Prusky & Plumbley 1992, Peres 1998).
92 *Colletotrichum musae* has long been considered the only species associated with banana
93 anthracnose in Brazil. However, the historical reliance on host range to define species limits,
94 such that any *Colletotrichum* species associated with banana is determined on that basis to be
95 *C. musae*, reduces the utility and reliability of this assertion. The diversity of *Colletotrichum*
96 species and other phytopathogenic fungi in Brazil found in association with crops such as
97 mango, papaya, grape, and coconut (Lima *et al.* 2013, Marques *et al.* 2013a, b, Netto *et al.*
98 2014, Vieira *et al.* 2014, Rosado *et al.* 2016), and the diversity of species found in association
99 with banana in other countries (see above) leads us to suspect that a diverse suite of
100 *Colletotrichum* species may also be associated with banana anthracnose in Brazil. The
101 reported variation in epidemiological factors among species, such as differential virulence
102 across cultivars (Lima *et al.* 2014), highlights the importance of determining the diversity of
103 species associated with banana anthracnose in Brazil and is prerequisite to developing optimal
104 management strategies for the disease (Phoulivong 2011).

105 In addition to host association, identification of *Colletotrichum* has traditionally been
106 based on morphological and cultural characteristics (von Arx 1957, Sutton 1980, 1992, Bailey
107 & Jeger 1992, Freeman *et al.* 1998) and more recently on the basis of multilocus phylogenetic
108 inference. However, the integration of data among studies in order to build a comprehensive
109 understanding of the phylogeny and taxonomy of *Colletotrichum* is limited by the lack of a
110 standardized approach for data collection. For example, while limited morphological variation
111 inhibits the utility of taxonomy based on phenotypic characters alone, the reliability of these
112 characters for differentiating existing species is difficult to assess due to variation induced by
113 cultural conditions (e.g. media and temperature) among studies (Cannon *et al.* 2000, Cai *et al.*
114 2009, Rojas *et al.* 2010, Weir *et al.* 2012, Doyle *et al.* 2013, Yan *et al.* 2015). Cai *et al.*
115 (2009) chose potato dextrose agar (PDA) as the standard for morphological studies of
116 *Colletotrichum*, but other culture media, such as corn meal agar (CMA) and Synthetischer
117 nährstoffarmer agar (synthetic nutrient-poor agar - SNA) continue to be used. We believe a
118 standard medium should be chosen on the basis of objective criteria, such as minimizing the
119 phenotypic variance within isolates to potentially maximize differences among species.
120 Similarly, there is a lack of consensus on the molecular data that should be collected for the
121 identification or description of new *Colletotrichum* species. While many studies include
122 sequence data from actin (ACT), chitin synthase (CHS-1), β-tubulin (TUB2), calmodulin
123 (CAL), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS),
124 and the nuclear ribosomal internal transcribed spacer (ITS) (e.g. Prihastuti *et al.* 2009, Wikee
125 *et al.* 2011, Weir *et al.* 2012, Lima *et al.* 2013, Udayanga *et al.* 2013, Huang *et al.* 2013, Yan
126 *et al.* 2015), sequence data from DNA lyase (Apn2) and the intergenic region between the
127 Apn2 and MAT1-2-1 genes (Apn2/MAT-IGS) have been used in several recent studies and
128 shown to contain strong phylogenetic signal within the *C. gloeosporioides* species complex
129 (Rojas *et al.* 2010, Silva *et al.* 2012a, Silva *et al.* 2012b, Doyle *et al.* 2013, Sharma *et al.*
130 2013, 2015, Vieira *et al.* 2014, Liu *et al.* 2015). Similar to the selection of standards for

morphological study, we need to use objective criteria to select genomic regions for taxonomic and evolutionary studies of *Colletotrichum* in order to make robust phylogenetic inferences and understand how these choices impact our results.

There is also a need to understand how the vagaries of data processing affect inferential reliability in recently diverged and speciose lineages such as the *Colletotrichum gloeosporioides* complex. The accurate estimation of nucleotide homology through multiple sequence alignment (MSA) is critical for all comparative sequence analyses that use an MSA as input (Sela *et al.* 2015). However, accurate MSA estimation and the assignment of positional homology across an alignment is not trivial (Wu *et al.* 2012) and alignment method can have a larger impact than inference method on the resulting topology (Morrison & Ellis 1997, Landan & Graur 2007). However, identifying and filtering those columns in the alignment that are sensitive to alignment method is one approach to remove alignment noise and obtain reliable phylogenetic estimates (Wu & Scott 2012). Alignment adjustment is commonly done by manual inspection, with the goal of identifying and correcting regions where the MSA program seems to have introduced errors (Hyde *et al.* 2013). The principle problem with manual alignment adjustment is setting guidelines on how to do it can be difficult or impossible such that it is replicable. However, a number of software packages have been developed with a diversity of approaches aimed at detecting unreliable alignment columns such as AliScore (Kück *et al.* 2010), Guidance (Penn *et al.* 2010, Sela *et al.* 2015), GBLOCKS (Castresana 2000, Talavera & Castresana 2007), NOISY (Dress *et al.* 2008), TrimAL (Capella-Gutiérrez *et al*. 2009) and Zorro (Wu *et al.* 2012). The introduction of alignment errors can potentially have a significant impact on *Colletotrichum* taxonomy if care is not taken to adopt alignment approaches that effectively maximize detection and removal of unreliable alignment residues, yet minimize the removal of phylogenetically informative sites.

In this study, we set out (1) to compare two commonly used alignment filtering algorithms for their ability to retain phylogenetic information while limiting potentially spurious alignment residues, (2), to assess the relative informativess of existing phylogenetic markers for *Colletotrichum* systematics and determine the impact of marker choice on the estimation of concordance factors in a Bayesian concordance analysis and (3) to determine the impact of culture medium on morphological variance in the *C. gloeosporioides* species complex and its impact on species discrimination. We then apply these insights to confidently reconstruct the phylogenetic relationships among the *Colletotrichum* species from banana in Brazil and compare the pathogenicity and levels of virulence of these species across different banana cultivars in order to understand their potential as pathogens in the field.

MATERIALS AND METHODS

Sampling and isolation

We collected samples from banana growing areas distributed among 11 Brazilian states between March 2012 and August 2013: Alagoas (1 city), Bahia (2), Distrito Federal (3), Espírito Santo (1), Goiás (2), Minas Gerais (3), Para (1), Paraná (1), Pernambuco (6), Santa Catarina (5) and São Paulo (4). The following banana cultivars were sampled: 'Ouro' (diploid *Musa acuminata*, AA group); 'Nanica', 'Nanicão' 'Caturra', 'Caru-Roxa', 'D'água' (triploid *M. acuminata*, AAA group, subgroup Cavendish); 'Prata', 'Pacovan', 'Maçã' (triploid *Musa* × *paradisiaca*, AAB group, subgroup Prata); and 'Terra' (triploid *Musa* × *paradisiaca*, AAB group, subgroup Plantain). One unripe fruit was collected from a minimum of 10 plants in each orchard with each sampling point separated by at least 20 meters. Fruits were returned to the lab, washed in running water and kept in a humid chamber for two days. The fruit were then removed from the humid chamber and maintained in the lab and monitored for the

appearance of lesions. Conidia from developing acervuli were transferred to Petri dishes containing potato-dextrose-agar (PDA – Himedia, India). Single conidium isolates were preserved on PDA slants at 4 °C and in cryogenic tubes containing autoclaved distilled water at room temperature.

Selection of Isolates for genetic analysis

In order to select isolates encompassing the range of diversity in our collection to include in the phylogenetic analysis, we first separated the isolates into morphological groups. The isolates were grown on PDA in Petri dishes at 25 °C under continuous fluorescent light. After seven days, we split the isolates into two groups based on an assortment of characters including growth rate, colony color, and the texture of aerial mycelia. Group 1 isolates were similar in morphology to the description of *C. musae* (Su *et al.* 2011, Weir *et al.* 2012), while Group 2 included all other isolates of *Colletotrichum*. Isolates representing the geographic and host breadth of the sampling and morphological diversity were chosen for the phylogenetic analysis.

Types and representative cultures were deposited at the Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes" (CMM), with corresponding dried cultures deposited at the Pe. Camille Torrand herbarium (URM).

DNA extraction, PCR and sequencing

We grew the selected isolates on potato dextrose (PD) broth for seven days and mycelia were separated from the culture medium by filtration prior to genomic DNA extraction. We extracted genomic DNA using the CTAB (cetyl trimethyl ammonium bromide) protocol described by Doyle & Doyle (1990) with slight modifications. DNA

208 concentration measurements were done on the NanoVue Plus Spectrophotometer (GE
209 Healthcare, USA) and diluted to 25 ng/μL for PCR reactions.

210 We performed the PCR amplifications in a 12.5 μL volume reaction containing 4 μL
211 of PCR-grade water, 1 μL of DNA template, 0.625 μL of each primer (10 μM), and 6.25 μL
212 of 2X PCR Master Mix (Promega GoTaq® Master Mix, Wisconsin, USA). The volume of the
213 PCR reaction was increased to 25 uL for loci requiring internal sequencing primers.

214 We first sequenced the intergenic spacer between the 3' end of the DNA lyase and the
215 mating type locus MAT1-2 (APN2/MAT-IGS) for all selected isolates. This marker was used
216 to identify the isolates belonging to the *C. gloeosporioides* species complex and to estimate
217 haplotype diversity among the isolates with DnaSP 4.0 (Rozas *et al.* 2003). Isolates
218 representing the range of genetic diversity based on the haplotype analysis were selected to
219 amplify an additional eight loci: actin (ACT), calmodulin (CAL), chitin synthase (CHS-1),
220 DNA lyase (APN2), nuclear ribosomal internal transcribed spacer (nrITS), β-tubulin (TUB2),
221 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glutamine synthetase (GS). The
222 APN2/MAT-IGS region was amplified and sequenced using primers CgDL_F6 and
223 CgMAT1_F2 (Rojas *et al.* 2010); ACT with ACT-512F and ACT-784R (Carbone & Kohn
224 1999); CAL with CL1C and CL2C (Weir *et al.* 2012); CHS-1 with CHS-79F and CHS-345R
225 (Carbone & Kohn 1999); APN2 with CgDL_R1 and ColDL_F3 (Rojas *et al.* 2010); ITS with
226 ITS1 (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990); and TUB2 with T1 and T22
227 (O'Donnell & Cigelnik 1997), along with the internal sequencing primers Coll_Bt_F1int and
228 Coll_Bt_R1int (Rojas *et al.* 2010). New primers were designed for the amplification of the
229 GAPDH and GS loci as described below. In order to reduce the proportion of missing data
230 and improve the ability to differentiate closely related species, we also sequenced some loci
231 for representative isolates of species belonging to *C. siamense sensu lato* from the Rojas *et al.*
232 (2010), Doyle *et al.* (2013), Lima *et al.* (2013) and Vieira *et al.* (2014) studies.

The GAPDH locus is among the more informative markers for resolving *Colletotrichum* species (Cai *et al.* 2009, Hyde *et al.* 2014). However, the primers commonly used to amplify this region produce a 250 – 300 bp fragment. In contrast, fragments of 400—1 250 bp are amplified for other genera such as *Alternaria*, *Stemphylium*, *Ulocladium* and *Phaeosphaeria* (Câmara *et al.* 2002, Ueng *et al.* 2003, Inderbitzin *et al.* 2009, Runa *et al.* 2009). In order to recover additional phylogenetic information from this locus, we developed and tested a novel set of primers to generate longer GAPDH sequences. The GAPDH gene (1 233 bp), an intergenic region of 1 306 bp, and the first exon of a hypothetical protein (2 330 bp) was excised from scaffold 129 (position 50814 to 55682) of *C. gloeosporioides* Nara gc5 (GenBank accession number KB020480). This fragment was aligned with *C. gloeosporioides* (M93427), *C. graminicola* (GG697347), *C. higginsianum* (CACQ02005624) and *C. orbiculare* (KB725678) genome sequences, and new primers were designed with Primer3 (Untergasser *et al.* 2012) as implemented in Geneious 8.1 (Auckland, New Zealand) in the conserved regions. The first set of primers, GAP-95 (5'-CCGTCAACGACCCCTTCATT-3') and GAP-1174 (5'-AACCCCACTCGTTGTCGTAC-3'), amplify the 3' end of the GAPDH exon 1, the intron, and part of the second exon. The second set of primers, GAP-1041 (5'-CTACACCGAGGACGATGTCG-3') and GAP/IGS-2044 (5'-TTCTACGGGAAAACCAGGGC-3'), amplify the end of the second exon and a portion of the 5' end of the intergenic spacer (GAP2-IGS) between the GAPDH gene and a hypothetical protein.

The standard GS primers (Stephenson *et al.* 1997) sequenced poorly for some isolates due to a thymine homopolymer near the forward primer binding site. Weir *et al.* (2012) designed a novel set of primers to amplify this locus, but in our experience these generate multiple non-specific products. In order to address this problem, we designed new primers: GS-64F (5'-CCGGAGAATYCTTTWCACGA-3') and GS-967R (5'-CTTCAGGTAGACGTCAGAGTTG-3'). The new GS primers were designed in conserved

259 regions across an alignment of GS sequences from *C. horii* ICMP10492 (GenBank accession
260 number JX010137), *C. hymenocallidis* CBS125378 (JX010100), *C. jasmini-sambac*
261 CBS130420 (JX010105), *C. kahawae* IMI319418 (JX010130), *C. musae* CBS116870
262 (JX010103), *C. nupharicola* CBS470.96 (JX010088), *C. psidii* CBS145.29 (JX010133), *C.*
263 *salsolae* ICMP19051 (JX010093), C. *siamense* ICMP18578 (JX010094), *C. theobromicola*
264 CBS142.31 (JX010064), *C. theobromicola* CBS129945 – syn. *C. fragariae* (JX010139), and
265 *C. gloeosporioides* Nara gc5 (XM007281411).

266 We performed PCR reactions with the following cycling parameters: APN2 and
267 APN2/MAT-IGS – initial denaturing for 3 min at 95 °C, followed by 35 cycles of 95 °C for
268 30 s, 62 °C for 45 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min;
269 ACT and CAL – initial denaturing for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30
270 s, 57 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min; ITS
271 and CHS-1 – initial denaturing for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 55
272 °C for 30 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min; GAPDH
273 and GAP2-IGS – initial denaturing for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30
274 s, 58 °C for 30 s, and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min;
275 TUB2 – initial denaturing for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 53 °C
276 for 30 s, and 72 °C for 1 min and 30 s, followed by a final extension at 72 °C for 10 min. PCR
277 products were purified and sequenced on an ABI 3730*xl* (Applied Biosystems) at Beckman
278 Coulter Genomics (Danvers, Massachusetts, USA).

279 In order to confirm the identity of the isolates classified as *C. musae* (Group 1) but not
280 included in the phylogenetic analysis, we designed a set of primers intended to be specific to
281 *C. musae*. The forward primer Musae_tub_33F (5'-GGTACCCCGACATTATG-3') was
282 designed such that the 3' end of the forward primer spans a unique insertion in the TUB2
283 region of *C. musae*, and is used in combination with the reverse primer Musae_tub_387 (5'-
284 CAATGTGGCCAGATAGGGGG-3'). The PCR reactions were performed using the

following cycling parameters: initial denaturing for 3 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s.

Contig assembly, alignment and phylogenetic reconstruction

Sequence reads were assembled into contigs and edited in Geneious 8.1 (Kearse *et al.* 2012). Sequences of *Colletotrichum* ex-type and reference strains from previous phylogenetic studies were retrieved from GenBank and included in the analyses described below. Sequences generated in this study were deposited in GenBank (Table 1).

Initially, we estimated the multiple sequence alignments (MSAs) for each locus independently with the online implementation of MAFFT version 7 (Katoh & Toh 2013) using the G-INS-i iterative refinement method. Poorly aligned sequences were manually adjusted using MEGA 6.06 (Tamura *et al.* 2013). We also evaluated the performance of GUIDANCE2 (Sela *et al.* 2015) and GBLOCKS (Castresana 2000, Talavera & Castresana 2007), two automated methods of alignment filtering. GUIDANCE2 assigns a confidence score for each residue, column, and sequence in the MSA. It first produces a base MSA or accepts a base MSA as input. Neighbor-joining trees are then generated from bootstrapped datasets and each is used as a guide tree to generate a unique alignment. The set of MSAs generated from unique guide trees is then used to assess the sensitivity of the alignment to perturbations of the guide tree and assign scores to each residue-pair in the base MSA. The scores represent the proportion of MSAs where the pair is aligned together. GBLOCKS is another tool that selects conserved blocks of positions in the MSA as a function of the number of contiguous conserved positions, gaps, and conservation of flanking positions, resulting in the removal of poorly aligned and divergent regions.

In order to estimate alignment uncertainty and filter alignments with GUIDANCE2, the unaligned sequences were uploaded to the GUIDANCE2 server

311 (http://guidance.tau.ac.il/ver2/), and the alignment confidence scores were calculated using
312 the default parameters (MAFFT as the MSA algorithm; max-iterate = 0; pairwise alignment
313 method = 6mer; 100 bootstraps repeats). Two different alignments were generated: one,
314 hereafter referred to as trimmed, in which the columns with a confidence score below 0.93
315 were removed and another, hereafter referred to as masked, in which residues with scores
316 lower than the lowest cutoff were masked and treated as missing data. For removing uncertain
317 regions of the alignment using GBLOCKS, the alignments generated in MAFFT were
318 uploaded to the GBLOCKS server. Given the short size of some loci and the reduced number
319 of sequences in the alignments, we changed the default parameters (stringent selection) to
320 allow a less stringent selection of blocks, since the relaxed selection is better for short
321 alignments (Talavera & Catresana 2007).

322 We compared the three approaches employed for alignment filtering (GBLOCKS,
323 masked and trimmed by GUIDANCE2) with the manually edited alignment. The alignments
324 of each single locus and the concatenated matrix were compared according to the number of
325 characters, variable sites, and parsimony informative characters. The automated filtering
326 method that provided the highest values for all three parameters in the concatenated alignment
327 was chosen as the preferred approach, and was employed in all the subsequent phylogenetic
328 analyses.

329 In order to evaluate the topological impact of alignment filtering, the topological
330 distance among maximum likelihood trees of each combination of locus and alignment
331 filtering method were calculated as described below. The software TreeCmp v1.1-b308
332 (Bogdanowicz *et al.* 2012) was used to calculate the distances between the ML tree inferred
333 from the manually edited alignment (reference tree) and each of the ML trees inferred from
334 filtered alignments. Trees were compared by the Matching Split (MS) distance, which has
335 been shown to have better statistical properties than other metrics (Bogdanowicz & Giaro
336 2012), and the Robinson-Foulds (RF) distance (Robinson & Foulds 1981) which has been the

most commonly used distance metric since it was introduced. Identical topologies have a distance of zero for both metrics, however the RF distance can be maximized with a few minor rearrangements while the MS distance increases as the number of changes increases (Bogdanowicz & Giaro 2012) and is therefore considered to be more reliable for determining the degree of topological change among treatments.

To infer the phylogeny for each locus (ACT, APN2, APN2/MAT-IGS, CAL, CHS-1, GAPDH, GAP2-IGS, GS, ITS and TUB2) and the concatenated matrix, we used maximum likelihood (ML) and Bayesian (BI) approaches. The ML analyses were performed with GARLI v 2.01 (Zwickl 2006) using the High Performance Computational Resources at Louisiana State University (http://hpc.lsu.edu). The ML tree was estimated assuming the best-fit model selected according to Aikake's Information Criterion corrected for small sample sizes (AICc) in jModelTest2 v 2.1.6 (Guindon & Gascuel 2003, Darriba *et al.* 2012) for each locus. The best ML tree is the one with the highest likelihood among 50 independent search replicates, with each search replicate terminated after 20 000 generations without an increase of more than 0.01 log-likelihood units, and found at least twice. When the best tree with the same likelihood score and topology was not found more than once, the number of search replicates was increased to 100. Nonparametric bootstrapping was performed with 1 008 bootstrap replicates (16 processors, 63 replicates per processor), with the best tree among four ML searches retained per bootstrap replicate. SumTrees 3.12.0, part of the DendroPy Phylogenetic Computing Library version 4.0.3 (Sukumaran & Holder 2010), was used to map the node frequencies to the best trees.

Bayesian phylogenetic estimates were inferred with MrBayes v. 3.2.6 (Ronquist *et al.* 2012) implemented on the CIPRES cluster (https://www.phylo.org/portal2/home.action) using the best-fit models of nucleotide substitution selected according to AICc by MrModeltest 2.3 (Nylander 2004). Four parallel runs were conducted with one cold and three heated Markov chain Monte Carlo (MCMC) search chains per run for 10 M generations,

363 sampling every 1 000 generations. Convergence of all parameters was checked using Tracer
364 v. 1.5 (Rambaut & Drummond 2007) to ensure that ESS values were greater than 200 and all
365 parameter estimates converged across runs. Posterior probabilities (PP) were calculated after
366 discarding the first 25% of generations as burn-in. Alignments and tree files are available in
367 TreeBASE (Submission ID: XXXXX).
368
369 **Species delimitation**
370
371 In order to determine if a clade could be recognized as an independent evolutionary
372 lineage, we applied the genealogical concordance phylogenetic species recognition (GCPSR
373 *sensu* Dettman *et al.* 2003) approach. A clade was considered an independent evolutionary
374 lineage if it satisfied one of the two following criteria: genealogical concordance or
375 genealogical non-discordance. The genealogical concordance criterion was satisfied if the
376 clade was present in the majority of the individual gene trees. For the non-discordance
377 criterion, the clade must be well supported in both ML and BI analysis (≥70 and ≥0.95
378 respectively) of at least one gene, and not contradicted in any other individual gene tree at the
379 same level of support. Novel species were recognized if the clade was recognized as an
380 independently evolutionary lineage as described above, was resolved with significant support
381 in both ML and BI analysis for the 10-locus concatenated dataset, and was not nested within
382 clades containing the type of any previously described species in both analyses.
383
384 **Phylogenetic informativeness**
385
386 The phylogenetic information content of each marker was assessed with the online
387 application PhyDesign (Lopez-Giraldez & Townsend 2011), which implements an empirical
388 metric of phylogenetic informativeness. This program computes the amount of phylogenetic

389 information in a genomic region across the history of the group based on site-specific
390 evolutionary rates (Townsend 2007). The set of markers used for *Colletotrichum* species
391 delimitation in the majority of studies (ACT, CAL, CHS-1, GAPDH, ITS and TUB2), and the
392 APN2, APN2/MAT-IGS, GAP2-IGS and GS were compared for informativeness across
393 different epochs.

394 The primer set used by Rojas *et al.* (2010) and in the present work for TUB2 amplifies
395 a 1 400—1 450 bp region. However, most studies generate TUB2 sequences using the primers
396 Bt2a and Bt2b (Glass & Donaldson 1995), which amplify a 450—500 bp fragment, or T1
397 (O'Donnell & Cigelnik 1997) and Bt2b, which amplifies a 650—700 bp fragment. For the
398 GAPDH region, the sequences generated using the primers GDF and GDR (Templeton *et al.*
399 1992) amplify fragments of 260—300 bp, while the primers GAP-95 and GAP-1174,
400 designed in the present work, amplify fragments of 960—980 bp. In order to evaluate the
401 informativeness of these smaller regions, the GAPDH and TUB2 alignment were resized in
402 MEGA 6.06, trimming the sequences at the 3' end near each primer-binding site, and named
403 according to the final alignment length for the analyses: T1+T22 – TUB-1460; Bt2a+Bt2b –
404 TUB-444; T1+Bt2b – TUB-702; GDF+GDR – GAPDH-208; GAP-95+GAP-1174 –
405 GAPDH-972.

406 A separate ML estimate was inferred in GARLI 2.01 using a reduced alignment with
407 one isolate per species, including only species where all 10 loci were sequenced. The ML tree
408 was transformed to an ultrametric tree in R v. 3.2.2 (R Core Team 2016) using the 'chronos'
409 function in the ape package (Paradis *et al.* 2004). This tree and the partitioned alignment files
410 were used as input in PhyDesign. The final concatenated alignment comprised 13 partitions
411 representing the 10 sequenced loci. The informativeness profiles were generated according to
412 informativeness values calculated on a net and on a per site basis. The markers were then
413 ranked according to the following variables: length, variable and parsimony informative

414 characters, percentage of parsimony informative characters, maximum net and per site
415 informativeness values, and the time unit to reach the maximum informativeness values.
416 In order to estimate the proportion of markers supporting each clade, we estimated
417 primary concordance trees using the Bayesian concordance analysis (BCA) implemented in
418 BUCKy v. 1.4.4 (Ané *et al.* 2007, Larget *et al.* 2010). Bayesian concordance analysis,
419 agnostic about the causes of gene tree discordance (e.g. incomplete lineage sorting (ILS) vs.
420 hybridization), is a coelascent-based summary method that aims to reconcile gene trees to
421 estimate the species tree while integrating gene tree uncertainty by sampling from a posterior
422 distribution of topologies (Ané *et al.* 2007; Baum, 2007; Larget *et al.* 2010; Chung & Ané,
423 2011). This approach also allows for quantifying the proportion of the genome that supports a
424 particular node, expressed as a concordance factor (CF). In the case of BCA, both low levels
425 of phylogenetic signal as well as high levels of incomplete lineage sorting or hybridization
426 can result in low CFs for species recognized on the basis of GCPSR. Therefore, datasets with
427 increased phylogenetic signal should lead to an increase in CFs at those nodes where the lack
428 of phylogenetic information is the principle cause of low CFs as opposed to high levels of
429 gene tree discordance or incomplete lineage sorting. The dataset for the BCA analysis was
430 reduced to 28 isolates, which comprise representatives from different species. Only isolates
431 with all the loci sequenced were chosen for this analysis. The posterior distribution of
432 individual gene trees was inferred in MrBayes using the same model parameters for each
433 partition as in the previous Bayesian analysis with four runs, four MCMC chains per run, and
434 trees and parameter values sampled every 5000 generations. The preliminary concordance
435 analysis was run using the discordance parameters (α) of 0.1, 1 and 10 with two MCMC
436 chains of 1 M generations following a 25% burn-in to determine the sensitivity of the BCA
437 results to discordance parameter values. Given all the runs converged on the same primary
438 concordance tree with similar concordance factors, only the tree from the run with α=1 is
439 presented. Three primary concordance trees were estimated to evaluate the impact of marker

choice on phylogenetic inference using the following datasets: all 10 loci; the most commonly used loci [ACT, CAL, CHS-1, GAPDH, ITS and TUB2, hereafter called 'legacy genes' *sensu* Kepler & Rehner (2013)]; and the loci chosen as the best according to the informativeness profiles and alignment variables described above. The BCA trees were compared according to changes in the topology and CFs. Another primary concordance tree was estimated from legacy gene sequences of 58 isolates in order to incorporate more species in the analysis and determine the proportion of genes that resolve some of the *Colletotrichum* species currently recognized.

Finally, phylogenetic trees were inferred under the ML and BI criteria using the best loci and the same inference parameters as described for the previous multilocus phylogenetic analysis.

Characterization of colony characters and micromorphology

For studies of colony and conidial morphology, isolates were grown on PDA, corn meal agar (CMA), and Synthetischer nährstoffarmer agar (SNA) (Nirenberg 1976) in polystyrene Petri dishes and incubated at 23 – 25 °C with continuous fluorescent light (24h). Pieces of sterile filter paper were placed on the surface of the solidified CMA and SNA to induce conidiation.

The methodology used by Doyle et al (2013) was followed to calculate the growth rate. Five millimeter plugs were taken from the expanding margin of colonies grown for seven days on CMA, and transferred to the center of a 100 × 15 mm polystyrene Petri dishes with PDA. Each isolate was plated to three replicate plates and incubated as described above. Four radial measurements were taken from the edge of the plug to the margin of the colony every 48 hours over the course of four days, resulting in eight radial measurements per replicate. Each Petri dish was then digitized and the distance between markings was measured

466 to calculate the mycelial growth rate (mm/day). The cultures were kept under the same
467 incubation conditions,and the colony characters were recorded from 7-day-old colonies.
468 Morphological observations were made from cultures actively conidiating on PDA,
469 CMA, and SNA. Discs of filter paper were placed onto the culture media surface to induce
470 conidiomata development when necessary. Conidia, phialides, and occasionally perithecia and
471 ascospores were mounted in water for microscopic observation. Hyphal appressoria were
472 observed using slide cultures (Johnston & Jones 1997): a 10 mm^2 block of CMA was placed
473 on a sterile microscope slide, each of the four corners of the block was inoculated, covered
474 with a sterile coverslip, and incubated at 25 °C for seven days. Microscopic images were
475 made with an AxioCam MRc digital camera attached to a Zeiss Universal Transmitted light
476 microscope using phase contrast illumination. The pictures were captured using the
477 AxioVision version 4.8.10 software.
478 At least 25 measurements per structure were taken using ImageJ v1.48 software
479 (Schneider *et al.* 2012) and summary statistics calculated in R v. 3.2.2. Growth rates are
480 presented giving the extremes, average and standard deviation [(lower extreme-) average ±
481 standard deviation (-upper extreme)]. The other measurements also include the 1^{st} and 3^{rd}
482 quartiles, and are shown with the notation [(lower extreme-) 1^{st} quartile – average – 3^{rd}
483 quartile (-upper extreme)]. The R package ggplot2 (Wickham 2009) was used to generate
484 graphical plots.
485 In order to facilitate the adoption of a standard medium for morphological evaluation
486 of conidial size, we assessed the effect of three different media on conidial measurements and
487 calculated the variance for conidial length, width and length/width ratio. The null hypothesis
488 of equal mean length, width and length/width ratio among species was tested with a one-way
489 ANOVA. We tested the residuals of the dependent variables by media and measurement for
490 normality with the Shapiro-Wilk test and all non-normally distributed data were transformed
491 with the Box-Cox transformation using the maximum-likelihood estimate (MLE) of λ,

however the results with transformed or untransformed data did not differ. Pairwise differences among *Colletotrichum* species were determined for each culture medium and measurement by a Tukey honest significant difference (HSD) test at the 5% significance level. All statistical tests were done using R v. 3.2.2.

Pathogenicity and virulence tests

Representative isolates among the different species were chosen for the pathogenicity and virulence test. The inoculation was carried out on banana fruits of the cultivars 'Maçã', 'Pacovan', 'Prata' and 'Terra'. The fruits were washed in running water, surface disinfected in 1 % NaOCl for 3 min, then rinsed in sterile distilled water. After drying at room temperature, the fruits were placed in plastic trays on paper towels moistened with distilled water to increase humidity. Each fruit was placed on a sterilized Petri dish to avoid direct contact with water. Fruits were wounded at two points by pricking the surface with a pin to a depth of 3 mm. The fruits were inoculated with 20 µL of a conidial suspension (1×10^6 conida/mL) on the wounds, and fruits inoculated with distilled water were used as a negative control. The trays were enclosed in plastic bags and incubated at 25°C. The plastic bags and paper towels were removed after 48 h and the fruits were kept at 25°C. The isolates were tested in four replicates (two fruits per replicate).

Pathogenicity was determined four days after inoculation by observing the presence/absence of symptoms. The virulence was assessed by measuring the radial diameter of the lesions and represented by the mean of two diameters per fruit. Differences in virulence among *Colletotrichum* species were determined for each cultivar by a one-way ANOVA and means among species were compared by LSD test at the 5% significance level using the software Statistix v.10 (Statistix 2013).

RESULTS

Isolation and morphological screening

A total of 431 *Colletotrichum* isolates were differentiated into nine morphological types (morphotypes) according to differences in the color and texture of the colonies (Fig. 1). Four hundred and three isolates were of a single morphotype and assigned to Group 1, while the remaining 28 isolates were distributed among the other 8 morphotypes and assigned to Group 2. Twenty-four isolates from Group 1 representing the geographical and host cultivar range of the sampling and all 28 isolates from Group 2 were chosen to amplify and sequence the APN2/MAT-IGS region to assess haplotype diversity.

Molecular screening and multilocus phylogeny

Based on the haplotype analysis of the APN2/MAT-IGS sequences, there were 12 distinct haplotypes among the 52 isolates sequenced. The isolates belong to the *C. gloeosporioides* species complex based on blastn queries of the NCBI nucleotide database, the majority of which were most similar to species within the "Musae clade" (*sensu* Weir *et al.* 2012). Up to three isolates representing the range of geographic diversity of each haplotype were chosen to sequence the remaining loci.

The alignment filtering methods produced alignments that carried with respect to the number of parsimony informative characters. When compared with the manually edited alignments, all filtering approaches yielded alignments equal to or shorter in total length, number of variable characters, and number of parsimony informative characters (Table 2). GBLOCKS was the most stringent method, with a reduction in the size of the alignment, resulting in a combined dataset 6 041 bp in length and 873 parsimony informative characters.

In contrast, masking residues with GUIDANCE2 was the least stringent method, producing alignments with only slightly fewer variable and parsimony informative characters for most loci than the manual alignment and a concatenated alignment 8 141 bp in length with 982 parsimony informative characters. Since apparently more phylogenetically informative sites were retained in the alignments after masking with GUIDANCE2, alignments masked with GUIDANCE2 were used for all subsequent analyses.

The trees inferred from each of the filtered alignments were topologically distinct from the reference tree (manual alignment) in nearly all cases (Table 2). The trees most similar (smaller MS or RF values) to those inferred from the manually edited alignment varied for each locus. For example, the topology inferred from the ACT alignment with residues masked in GUIDANCE2 was most similar to the topology inferred from the manual alignment (MS = 5, R-F = 1.5), while for GAPDH the lowest tree distance was between the trees inferred from the manual alignment and that filtered with GBLOCKS (MS = 14, R-F = 1.5). However, the smallest topological distances were observed between the trees inferred from the manual alignment and those filtered with GUIDANCE2 (either trimmed or masked) in 7 of the 9 individual locus alignments where all three filtering methods produced a modified MSA (Table 2). Similarly, the trees inferred from the alignment of the concatenated data with residues masked in GUIDANCE2 were most similar (MS = 32, R-F = 8) to the tree inferred from the manual alignment.

The locus boundaries in the concatenated alignment of 108 *Colletotrichum* sequences were: ACT: 1—294, APN2/MAT-IGS: 295—1262, APN2: 1263—2091, CAL: 2092—2856, CHS-1: 2857—3155, GAPDH: 3156—4190, GAP2-IGS: 4191—5089, GS: 5090—6034, ITS: 6035—6634, TUB2: 6635—8141. The trees generated by the ML approach and BI for single genes datasets (S. Fig. 1-10) and the concatenated matrix (Fig. 2) were identical in topology, just varying with respect to the number of nodes and levels of node support.

569 Phylogenetic signal varied significantly among individual loci. Alignments of ACT,
570 CHS-1, and ITS yielded topologies with the lowest resolution with few well supported clades
571 in either the ML or BI analysis. Several described species represented by multiple isolates
572 were strongly supported as monophyletic in the trees inferred from CAL, GAPDH, GAP2-
573 IGS, GS and TUB2, with exception of those within *C. siamense s. l.* The trees inferred from
574 the APN2 and APN2/MAT-IGS alignments provided the best resolution and were the only
575 genes capable of resolving most of the species in the *C. siamense* species complex.
576 In contrast with most individual gene trees, the multilocus analysis resulted in a well-
577 resolved topology for both the ML and BI analysis. Most species were strongly supported,
578 with the exception of *C. aenigma* and *C. dianesei* which were only strongly supported in the
579 BI analysis. The *Colletotrichum* isolates from banana were resolved in eight independent
580 lineages in the multilocus tree, some of which were not nested in a clade containing the ex-
581 type of a previously described species.
582
583 **Species assignment**
584
585 *Colletotrichum* isolates from banana were assigned to four previously described
586 species using the GCPSR criteria. Eight isolates were nested within the C. *musae* clade and
587 one within the *C. tropicale* clade in the multilocus analysis with the maximum level of
588 support for both ML and BI analyses. The *C. tropicale* clade was recovered in most of the
589 single gene trees with strong support in at least one of the analyses, while *C. musae* clade was
590 strongly supported in all individual gene trees. Four isolates from banana were assigned to the
591 species *C. dianesei*.. The *C. dianesei* clade was well supported only the BI analysis of the
592 multilocus MSA. However, all isolates belonging to this species were placed together in a
593 strongly supported clade in the GS tree and was not contradicted in any of the other individual
594 gene trees. We are here reporting *C. musae* in association with banana anthracnose in Brazil,

595 and reporting the first noted association of *C. dianesei* and *C. tropicale* with banana
596 anthracnose worldwide.

597 Four lineages did not cluster with any previously described species, and one of them is
598 described in the present work as a novel species. This lineage is described in the Taxonomy
599 section below as *Colletotrichum chrysophilum sp. nov*. *Colletotrichum chrysophilum sp. nov.*
600 is closely related to *C. fructicola* and *C. nuphar*icola and was strongly supported in both the
601 ML and BI multilocus analyses. This lineage includes isolates originally assigned to *C.*
602 *ignotum* by Rojas *et al* (2010) and placed in synonymy with *C. fructicola* by Weir *et al.*
603 (2012). This lineage is clearly separated from *C. fructicola* in the multilocus analysis,
604 genealogically concordant across seven loci, and strongly supported in four of them with no
605 strongly supported topological discordance. The isolates CMM4244, CMM4248 and
606 CMM4247 appear to represent three distinct lineages within *C siamense sensu lato*. Although
607 these isolates are are not nested within clades containing previously described species, we are
608 not describing them here given each one is represented by a single isolate and they are within
609 *C. siamense s.l.* which may represent a single species (see Liu *et al*. 2016).

610 The isolate CMM4242 from banana belongs to the clade containing the isolate
611 CBS142.31, which was designated the type of *C. fragariae*, a species placed in synonymy
612 with *C. theobromicola* by Weir *et al* (2012). In our multilocus analysis, the type of *C.*
613 *theobromicola* grouped with another isolate in a well-supported clade. Although the clade
614 containing the type of *C. fragariae* was only supported by the BI analysis of the 10 locus
615 concatenated dataset, these isolates are clearly distinct from *C. theobromicola* based on the
616 APN2 and APN2/MAT-IGS analyses. Both clades were well supported in the ML and BI
617 analyses in these two individual gene trees, and not contradicted by any other individual gene
618 tree. Given the two clades can be recognized as independent evolutionary lineages, we are
619 here resurrecting *C. fragariae* as a valid species.

620 The isolates CBS133251 and CBS133123, first described as *C. melanocaulon*, form a
621 well-supported clade distinct from *C. dianesei* in the multilocus analyses. *Colletotrichum*
622 *melanocaulon* was recently placed in synonymy with *C. dianesei* by Vieira *et al.* (2014).
623 However, this clade was recovered in the GAP2-IGS analysis with strong support in both ML
624 and BI and from the APN2 and GS analysis with strong support in the BI indicating it is an
625 independent evolutionary lineage from *C. dianesei*. However, *C. melanocaulon* is represented
626 by only two isolates from the same geographic region with identical haplotypes. Therefore,
627 given recent work suggesting *C. siamense s. l.* may be a single lineage (Sharma *et al.* 2013,
628 2015, Liu *et al.* 2016) and a need for taxonomic stability until better sampling can be
629 achieved, we elect not to resurrect *C. melanocaulon* as a valid species at this time.
630 Finally, the species-specific primers developed here were useful for distinguishing *C.*
631 *musae* from other related species. The species specific primers produced a single amplicon of
632 approximately 350 bp for all isolates identified as *C. musae* in the multilocus phylogenetic
633 analysis while all other species did not amplify (S. Fig. 11). All the remaining isolates from
634 morphological Group 1 also amplified with this set of primers, confirming their identity as *C.*
635 *musae*. Therefore, 93.5% of the isolates collected for this study are *C. musae* (403 isolates),
636 4.4% are *C. chrysophilum* (19 isolates), and the other species comprised the remaining 2.1%
637 (9 isolates).
638
639 **Phylogenetic informativeness**
640
641 The informativeness profiles estimated by PhyDesign are illustrated in Fig. 3 and the
642 informativeness values for each marker are summarized in Table 3. The maximum net
643 informativeness values (NIV) ranged from 9.81 to 167.57. APN2/MAT-IGS had the highest
644 NIV. GAP2-IGS, APN2, and the longest TUB2 alignment also had high and very similar
645 NIVs. CAL, GAPDH, and GS had intermediate NIVs that peaked between 0.25 and 0.27

time units. The maximum NIV of ACT, CHS-1, and ITS ranged between 9.81 and 24.81, and these markers presented nearly flat informativeness profiles. Based on the NIVs, APN2, APN2/MAT-IGS, CAL, GAPDH, GAP2-IGS, GS and TUB2 were more phylogenetically informative than ACT, CHS-1 and ITS.

The phylogenetic informativeness (NIV) of the GAPDH and TUB2 regions are directly related to the size of the fragments. Other variables such as the number of variable characters and parsimony informative characters were also correlated with NIV, all of which increased with the increasing length of the amplicon. According to the NIVs, the GAPDH-972 and TUB2-1460 are the amplicon sizes that are the most phylogenetically informative.

When examined on a per site basis, the markers with long intergenic or intragenic regions were the most informative, which was consistent with the NIV. The informativeness of the different GAPDH and TUB2 amplicons was positively correlated with the percentage of parsimony informative characters. GAPDH-208 had the highest per site informativeness value (PSIV) among the GAPDH amplicons and ranked second among all according to PSIV. In contrast, the three different TUB2 amplicons had almost the same PSIV.

The concordance factors (CF) on the primary concordance (PC) trees estimated in the BCA (Fig. 4) was affected by the choice of loci. In comparison with the 10 locus PC tree (Fig. 4A), CF values decreased when only the legacy genes were included in the BCA (Fig 4B), particularly for the *C. siamense s. l.* clade. In contrast, the CF values increased significantly on the branches subtending species-level lineages as well as those representing deeper level relationship when only the most phylogenetically informative genes were included in the analysis (Fig. 4C).

The PC tree estimated from the legacy genes and including more *Colletotrichum* species (Fig. 5) had generally low CF values for several clades. The *C. siamense s. l.* clade is resolved just by one gene (CF=0.17) and is discordant with the tree inferred from the concatenated dataset (Fig 2). *Colletotrichum endomangiferae* was the single species within

672 the *C. siamense* species complex that was supported as monophyletic by more than a single
673 gene (CF=0.35). Most of the species outside of the *C. siamense* complex were supported as
674 monophyletic by at least half of the genes (CF≥0.51). However, the relationships among
675 species were not strongly supported. Because the genes that separate *C. fragariae* and *C.*
676 *theobromicola* were not included in this analysis, isolates of both species were placed together
677 in the same clade.

678 Finally, the multilocus analysis of the seven best genes (Fig. 6) yielded a topology
679 very similar to that of the 10 gene multilocus analysis (Fig. 2) with comparable levels of
680 support (slightly stronger at some nodes and slightly weaker at others). *Colletotrichum*
681 *fragariae* ICMP17099, Bra5 and Bra8, *C. gloeosporioides* f. *stylosanthis* CBS124251, *C.*
682 *jasmini-sambac* CLTA01 and *C. theobromicola* ICMP17895 were removed from this analysis
683 due to missing data. While most topological changes are minor between the two analyses, the
684 most significant change is at the base of the *C. siamense* species complex. The basal
685 divergence within this lineage is unresolved in the 10-locus dataset when zero-length
686 branches are collapsed, while inferences from the 7-locus dataset suggest *Colletotrichum* sp.
687 2, *Colletotrichum* sp. 3, *Colletotrichum* sp. 4, *C. siamense s.s.* and *C. jasmini-sambac* are
688 sister to the remaining isolates within *C. siamense s.l.*, albeit with weak support.
689 *Colletotrichum aenigma* and *C. dianesei* continue to be weakly supported by one or both ML
690 and BI analyses, in contrast with the *C. fragariae* clade which was strongly supported as
691 monophyletic in both analyses of the 7-locus dataset. Based on the strong support across the
692 topology, the seven best genes confidently resolve the species within the *C. gloeosporioides*
693 species complex as well evolutionary relationships among them.
694
695 **Characterization of colony characters and micromorphology**
696

Nine morphological types (Fig. 1) among all isolates were recognized based on differences in colony characteristics. The dominant species was *C. musae*, which differed from the other species by the circular colony, white abundant and floccose mycelia, well developed orange conidial masses (Fig. 1A), and highest growth rate (11.5 ± 0.4 mm/day) (Fig. 7).

Variation in conidial size was induced by differences in the culture medium (Fig. 8). The isolates of *Colletotrichum* sp. 1 and *Colletotrichum* sp. 3 did not produce conidia on SNA and were not included in the analysis. The variance in conidial dimensions produced on PDA ranged from 0.43 to 8.06 for the length and from 0.15 to 0.34 for the width. The variance on CMA ranged from 0.35 to 1.24 for the length and from 0.07 to 0.19 for the width and the variance on SNA ranged from 0.37 to 1.65 for the length and from 0.10 to 0.22 for the width.

The variance on conidial dimensions affected the number of *Colletotrichum* species that could be differentiated based on conidial length, width and length/width ratio (Table 4). When the mean conidial measurements from CMA were compared in pairwise combinations, 13 of 15 combinations were significantly different for all three measurements; on PDA, 10 of 15 combinations for length and length/width ratio, and 8 of 15 for width; and on SNA, 13 of 15 combinations for length, 10 of 15 for width, and 12 of 15 for length/width ratio. CMA was the medium upon which all of the six *Colletotrichum* species evaluated could be separated by the combination of conidial length and width.

Pathogenicity and virulence

All isolates tested were pathogenic on the four banana cultivars, resulting in fruit infection 4 days post-inoculation. The symptoms induced by all *Colletotrichum* species were similar, with sunken dark brown to black lesions. No symptoms were observed on the controls, which differed statistically from the other treatments in the LSD test ($p \leq 0.05$).

723 The virulence of each *Colletotrichum* species was dependent on the banana cultivar
724 (Fig. 9). *Colletotrichum musae*, *Colletotrichum* sp. 1, *Colletotrichum* sp. 2, and
725 *Colletotrichum* sp. 4 were the most virulent on the cultivars 'Maçã' and 'Pacovan'.
726 *Colletotrichum dianesei* was the least virulent species on the cultivar 'Pacovan', and together
727 with *C. fragariae* on the cultivar 'Maçã'. *Colletotrichum tropicale*, *Colletotrichum* sp. 1, and
728 *Colletotrichum* sp. 4 produced the largest lesions on cv. 'Prata', while *C. dianesei* produced
729 the smallest lesions. *Colletotrichum fragariae* was significantly less virulent than all other
730 species on cv. 'Terra'. In general, *C. musae*, *Colletotrichum* sp. 1, and *Colletotrichum* sp. 4
731 were the most virulent on banana fruits, while *C. dianesei* and *C. fragariae* were the least
732 virulent.
733
734 **Taxonomy**
735
736 ***Colletotrichum chrysophilum*** W.A.S. Vieira, W.G. Lima, M.P.S. Câmara & V.P. Doyle **sp.**
737 **nov.** MycoBank: _____. Fig. 10
738
739 *Etymology:* The specific epithet refers to the association with the banana cultivar 'Ouro'
740 (gold) and is from the Greek chryso-, meaning gold, and –philus meaning -loving.
741
742 *Holotype*: **Brazil**, São Paulo, Registro, on *Musa* sp. cultivar 'Ouro', coll. W. G. Lima, Jul.
743 2013, URMXXXX, holotype living culture URM7368, CMM4268.
744
745 *Colonies* on PDA at first light grey, becoming dark grey, reverse light grey, growth rate at 25
746 ºC (6.2–)6.5—6.6—6.9(–7.2) mm/day (av. 6.7 mm/day, n = 12). Aerial mycelium grey, felt-
747 like, with orange conidial masses. *Sclerotia* absent. *Acervuli* on filter paper, dark brown,
748 present in culture. *Setae* on filter paper, abundant, long, dark brown, smooth-walled, two or

749 more septa, base truncate, tip ± acute. *Conidiophores* hyaline, smooth-walled to verruculose,
750 aseptate, unbranched. *Conidiogenous cells* hyaline, smooth-walled, cylindrical to
751 ampulliform, often extending to form new conidiogenous loci. *Conidia* formed from acervuli,
752 one-celled, smooth-walled, hyaline, cylindrical with rounded ends, sometimes oblong,
753 contents appearing granular. *Conidia* on PDA (12.8–)15.5—16.1—17.1(–20.5) × (4.5–)5.2—
754 5.4—5.7(–6.3) µm (av. 16.5 × 5.4 µm, n = 30), length/width ratio (2.3–)2.8—3—3.2(–4) µm
755 (av. 3.1 µm, n = 30). *Conidia* on CMA (13.4–)15.4—15.7—16.3(–17.9) × (3.7–)4.3—4.7—
756 4.9(–5.3) µm (av. 15.8 × 4.6 µm, n = 30), length/width ratio (2.8–)3.2—3.3—3.8(–4.4) µm
757 (av. 3.46 µm, n = 30). *Conidia* on SNA (13.5–)15.9—16.6—16.9(–18.3) × (4.7–)5—5.3—
758 5.6(–6.4) µm (av. 16.4 × 5.4 µm, n = 30), length/width ratio (2.4–)2.9—3.1—3.3(–3.8) µm
759 (av. 3.1 µm, n = 30). *Appressoria* in slide cultures, single, medium to dark brown, smooth-
760 walled, clavate, sometimes with undulate margin, (5,7–)7.3—8.6—10.3(–13.3) × (4.6–)4.9—
761 5.4—6.1(–7.3) µm (av. 9 × 5.5 µm, n = 25). *Perithecia* formed on SNA after about 10 d, dark-
762 walled, globose with long and wide ostiolar neck. *Ascospores* (11.8–)12.6—13.1—113.6(–
763 14.4) × (3.1–)3.4—3.6—3.8(–4) µm (av. 13 × 3.6 µm, n = 15).
764
765 *Geographic distribution and host range*: Known from *Musa* sp. from São Paulo, Brazil.
766 Originally reported as an endophyte from *Theobroma* and *Genipa* in Panama (Rojas *et al.*
767 2010), and from *Terpsichore* (current name: *Mycopteris*) in Puerto Rico (Doyle *et al.* 2013).
768
769 *Notes*: Rojas *et al.* (2010) described the species *C. ignotum*, which was later placed in
770 synonymy with *C. fructicola* by Weir *et al.* (2012). The types of *C. ignotum* and *C. fructicola*
771 belong to the same haplotype subgroup described in Rojas et al (2010). However, the second
772 haplotype subgroup comprises other isolates first described as *C. ignotum* and the isolate from
773 banana fruit. This same subgroup was denoted "*C. ignotum* 2" by Doyle *et al.* (2013).
774

775 *Genetic identification*: ACT, APN2, APN2/MAT-IGS, CAL, GAP2-IGS, GAPDH, GS and
776 TUB2 sequences separate *C. chrysophilum* from *C. fructicola*.
777
778 *Specimens examined*: **Brazil**, Minas Gerais State, on *Musa* sp. fruit rot, coll. W. Lima, Jul.
779 2013 (CMM4292 and CMM4387); **Brazil**, Pernambuco State, on *Musa* sp. fruit rot, coll. W.
780 Lima, Jul. 2013 (CMM4309 and CMM4352); **Brazil**, Santa Catarina State, on *Musa* sp. fruit
781 rot, coll. A. Reis, 2014 (CMM4363 and CMM4394).
782
783 ***Colletotrichum fragariae*** A.N. Brooks, Phytopathology 21: 113. 1931.
784
785 *Genetic identification*: APN2 and APN2/MAT-IGS clearly separate *C. fragariae* from *C.*
786 *theobromicola.*
787
788 *Notes*: The name *C. fragariae* was originally applied to Colletotrichum isolates associated
789 with a disease of strawberry (*Fragaria* × *ananassa*) runners (stolons) and petioles in Florida
790 (Brooks 1931). The type of *C. fragariae* (CBS 142.31) was later designated by Buddie *et al.*
791 in 1999.
792 Weir *et al.* placed *C. fragariae* in synonymy with *C. theobromicola* based on a
793 multilocus analysis of ACT, CAL, CHS-1, ITS, GAPDH, GS, SOD2 (manganese-superoxide
794 dismutase) and TUB2. However, Sharma *et al.* (2013) showed that sequences of APN2/MAT-
795 IGS, not analyzed by Weir *et al.* (2012), clearly support *C. fragariae* and *C. theobromicola* as
796 two monophyletic lineages, and the same result was found in our analysis (Fig. S3). The
797 sequences of APN2 can also separate both species (Fig. S2).
798
799
800

DISCUSSION

Diversity of *Colletotrichum* on Banana in Brazil

Colletotrichum musae has long been considered the sole causal agent of banana anthracnose (Meredith 1960, Su *et al.* 2011, Thangamani *et al.* 2011). However, Udayanga *et al.* (2013) reported one undescribed species within *C. siamense s. l.* associated with banana anthracnose in Thailand and *Colletotrichum gloeosporioides s. l.* was reported from Malaysia (Intan Sakinah *et al.* 2014), although species designation in the latter is questionable due to poor taxon sampling. These sequences were not nested within any of the eight lineages of *Colletotrichum* we found in association with anthracnose on 10 banana cultivars collected across 11 Brazilian states. Therefore, there are now 10 lineages within the *C. gloeosporioides* species complex known to be associated with banana anthracnose worldwide.

While multiple *Colletotrichum* species are associated with banana anthracnose in Brazil, *C. musae* is the most prevalent species, similar to the results of Udayanga *et al.* (2013). Most species within the *C. gloeosporioides* species complex that have been collected across a broad geographic distribution appear to be generalists, but no multi-locus phylogenetic studies have found *C. musae* in association with any other host species (Phoulivong *et al.* 2010, Udayanga *et al.* 2013, Sharma *et al.* 2015), suggesting that it may be host specific. This may be why *C. musae* is so prevalent on banana; however whether it is the most prevalent species associated with banana anthracnose on a global basis is not yet known. Global sampling of *Colletotrichum* in association with banana will help to address the question of whether *C. musae* is host specialized or its widespread distribution is simply the result of human-mediated dispersal. The species-specific primers we have developed here for the rapid detection of *C. musae* will greatly assist with this effort by reducing the cost associated with sequencing all isolates.

826 *Colletotrichum dianesei*, *C. fragariae* and *C. tropicale* represented a small percentage
827 of the species causing banana anthracnose and have also been reported from *Mangifera indica*
828 and *Coffea* spp., crops frequently cultivated alongside banana in Brazil (Lima *et al.* 2013,
829 Vieira *et al.* 2014, Silva *et al.* 2012a). These same species, and isolates of *C. chrysophilum*
830 (originally identified as C. *ignotum*), have also been reported from other regions in
831 association with plants often found in banana fields in Brazil, including *Annona muricata*,
832 *Cocos* sp., *Theobroma* spp., *Bauhinia variegata*, *Persea americana*, and *Psidium* sp. (Rojas *et*
833 *al.* 2010, Sharma *et al.* 2015). This suggests these species and the three undescribed lineages
834 collected in this study are incidental colonists of *Musa spp.* or recently jumped from other
835 hosts to banana and have not had time to proliferate despite the fact that they are as virulent or
836 more so based on our pathogenicity tests (Fig. 9). However, this is a question that needs to be
837 addressed with both more intensive and extensive sampling of alternate host species in
838 proximity to areas of banana cultivation.
839
840 **Methodological Perspectives on *Colletotrichum* Systematics**
841
842 Developing objective standards for data collection and analysis are particularly
843 important for studying the biology of a group of organisms as diverse, broadly distributed,
844 and economically important as *Colletotrichum.* As a community, we continue to
845 incrementally and independently add to the growing body of knowledge on *Colletotrichum*
846 systematics, but we hope to someday be able to collate all of the information that has been
847 collected into a comprehensive phylogeny and taxonomy of the group. However, such
848 standards that will enable the collation of data among studies have yet to be realized. Our
849 objective here was to evaluate some of the aspects of data collection and analysis that we
850 believe to be important for eventually reaching this goal. We have focused on alignment
851 methods in the interest of improving replicability across studies and improving the reliability

852 of phylogenetic inferences, phylogenetic informativeness of the most common markers
853 employed for phylogenetic analysis to aid with marker selection for future phylogenetics and
854 species delimitation studies, and morphological study of conidia in order to objectively
855 determine the optimal medium for characterizing and differentiating species.

856 Manual adjustments made "by eye" to multiple sequence alignments has been standard
857 practice in phylogenetics, however systematists are moving away from this approach towards
858 methods that are more replicable and account for alignment uncertainty (Ebersberger *et al.*
859 2012, Wu *et al.* 2012, Doyle et al. 2014, Tan *et al.* 2015). While accounting for uncertain
860 homology assessment during multiple sequence alignment is becoming commonplace in
861 molecular phylogenetics (Kuramae *et al.* 2006, Soria-Carrasco *et al.* 2007, Ebersberger *et al.*
862 2012), we are only aware of its application in a single study of *Colletotrichum* to date
863 (Jaramillo *et al.* 2015). The predominant use of alignment filtering has focused on lineages
864 with deep divergences because distantly related taxa are likely to have accumulated indels and
865 nucleotide substitutions making unambiguous alignment difficult (Wong *et al.* 2008).
866 However, phylogenetic inferences of recently diverged taxa or datasets with few
867 phylogenetically informative sites may also be topologically impacted by alignment
868 uncertainty (as opposed to choices of inference), as indicated by the topological differences
869 we see among trees inferred from different alignments (Table 2). Our results also indicate that
870 algorithmic approaches that trim alignments based on sequence conservation (or other fixed
871 attributes of the alignment), such as GBLOCKS, can remove many alignment residues that
872 can be reliably aligned and therefore unnecessarily dispense phylogenetically informative
873 sites when compared with filtering with Guidance (Table 2). We are not advocating moving
874 directly from alignment to phylogenetic analysis without first inspecting the alignment,
875 however if substantive manual adjustment must be made to an alignment, our results indicate
876 alignment choices can impact toplogical inferences and it is therefore advisable to adjust
877 alignment parameters or filter alignments to deal with alignment error rather than making

878 manual adjustments, such that other researchers can replicate a given study from start to finish
879 and phylogenetic inferences are more reliable and reproducible.

880 Marker selection for phylogenetic inference has become another topic of interest for
881 systematic biologists as focus has turned to the variation in reliability among loci for
882 phylogenetic inference at a time when phylogenomic scale data are becoming easier to collect
883 (Collins *et al.* 2005, Salichos and Rokas 2013, Betancur-R *et al.* 2013, Doyle *et al.* 2015).
884 While these empirical studies of marker reliability are contingent on partial *a priori*
885 knowledge of the true evolutionary history of a group, which is lacking in the *Colletotrichum*
886 *gloeosporioides* species complex, our results do highlight the impact that marker selection can
887 have on species delimitation (Fig. S1—S10, Fig. 4). This is particularly true for those studies
888 that delimit species by taking into consideration topological variation across independent gene
889 trees (i.e. GCPSR and coalescent-based methods). Accounting for variation among individual
890 gene trees allows for defining pools of recombination that are at the boundary between
891 reticulation and divergence in order to delimit species (Baum & Shaw 1995, Taylor *et al.*
892 2000, Dettman *et al.* 2003). However, as our results demonstrate, the choice of phylogenetic
893 markers can impact the detection of species boundaries. While application of the non-
894 discordance criterion allows for the recognition of lineages even when the majority of
895 phylogenetic markers have low levels of phylogenetic signal but little homoplasy, if only a
896 single gene with strong signal is included many lineages will remain obscured. This is
897 exemplified by the fact that some species can only be recognized by fewer than the majority
898 of individual gene trees and not always by the same genes. For example, *C. communis* and *C.*
899 *endomangiferae* are only distinct lineages in the APN2/MAT IGS gene tree (Fig. S3), while
900 *C. melanocaulon* was only distinguished from *C. dianesei* in both the BI and ML analyses of
901 GAP2 IGS (Fig. S6). Similarly, recognition on the basis of other criteria, such as the use of
902 concordance factors (CF) resulting from a Bayesian concordance analysis (BCA), is strongly
903 influenced by the signal in individual alignments (Figs. 4 and 5). Both conflicting

904 phylogenetic signal, whether due to ILS or hybridization, and lack of signal can reduce the CF
905 for a given lineage. Therefore, if CF thresholds are to be used to delimit species (Baum 2007,
906 Vialle *et al.* 2013), consideration should be given to both the suitability of the markers
907 selected for species recognition and whether these markers are representative of genomic
908 divergence. Markers with low signal, such as ITS, CHS, and ACT (Fig. 3), can drag down
909 concordance factors and obscure species boundaries in otherwise well defined lineages. If we
910 are ultimately to develop a comprehensive and stable taxonomy for the *Colletotrichum*
911 *gloeosporioides* complex that reflects the evolutionary history of the group, we will need to
912 dissect the phylogenetic signal of the markers being utilized and employ explicitly stated and
913 reproducible criteria for species recognition. This will be an ongoing effort as new markers
914 are developed and a phylogenomic perspective is brought to bear on evolutionary questions
915 within the group.

916 While nucleotide sequence data are the most reliable means of identifying species (e.g.
917 Liu *et al.* 2015, Pinho *et al.* 2015, Velho *et al.* 2015, Yan *et al.* 2015), the extent to which
918 phenotypic data are able to differentiate species is impossible to fully assess because of the
919 variation among studies with respect to incubation conditions. Both for taxonomic purposes
920 and because there is often a desire to inexpensively screen large numbers of isolates collected
921 during comprehensive surveys, we sought to objectively evaluate the utility of the three most
922 commonly used media for morphological characterization of conidia. Our results suggest the
923 best media to minimize the variance within species (Figure 8) and maximize among species
924 differences (Table 4) is CMA and SNA. However, we recommend using CMA over SNA for
925 two reasons. First, it is the only medium on which the mean conidial size was significantly
926 different across all species (Table 4) when including all dimensions (length, width, and
927 length/width ratio). Second, in our experience, conidia are consistently produced on CMA
928 while it is not always possible to induce conidiation on SNA (personal observation). The
929 primary drawback to using CMA as a standard medium is that it is not a defined medium (in

contrast to SNA), however we believe our results will be consistent across different manufacturers of CMA. While our comparisons were not exhaustive (all described species), we were able to compare several species associated with banana anthracnose across the principle media that have been used to characterize isolates within the *C. gloeosporioides* species complex in recent years. The adoption of CMA as the standard medium for studies of *Colletotrichum* will eventually allow us to compare all described species for morphological variation and perhaps differentiate some species based on morphology.

Conclusion

Molecular phylogenetics has been essential in providing novel insights into the diversity of *Colletotrichum* (Rojas *et al.* 2010, Damm *et al.* 2012a, b, Silva *et al.* 2012a, Weir *et al.* 2012, Doyle *et al.* 2013, Sharma *et al.* 2013, 2015, Vieira *et al.* 2014, Liu *et al.* 2015), principally in recognition of cryptic species in the species complex that could not be diagnosed based solely on classical phenotypic characters. However, our results indicate that the choice of phylogenetic markers can have a significant impact on the detection and delimitation of species boundaries and inference of the species tree from multiple gene trees. Similarly, our results demonstrate that phylogenetic signal and, therefore, topological inferences can be impacted by how individual alignments are curated. In this study, we have found several species of *Colletotrichum* across the major banana production areas of Brazil that were not previously known to be associated with anthracnose on banana fruit by applying alignment filtering approaches that retain the most phylogenetically informative sites using the most informative markers to delimit species on the basis of GCPSR. It is clear that the data we choose to collect and how we process these data can have an impact on our estimations of species diversity and evolutionary relationships within the *C. gloeosporioides* species complex. Therefore, while establishing a stable taxonomy and robust understanding of

956 *Colletotrichum* evolution depends on both improving sampling across host species and
957 geographic regions, a concerted effort to standardize and improve approaches to data
958 collection is also needed.
959
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979
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Yan J-Y, Jayawardena MMRS, Goonasekara ID, *et al.* (2015). Diverse species of *Colletotrichum* associated with grapevine anthracnose in China. *Fungal Diversity* **71**: 233–246.

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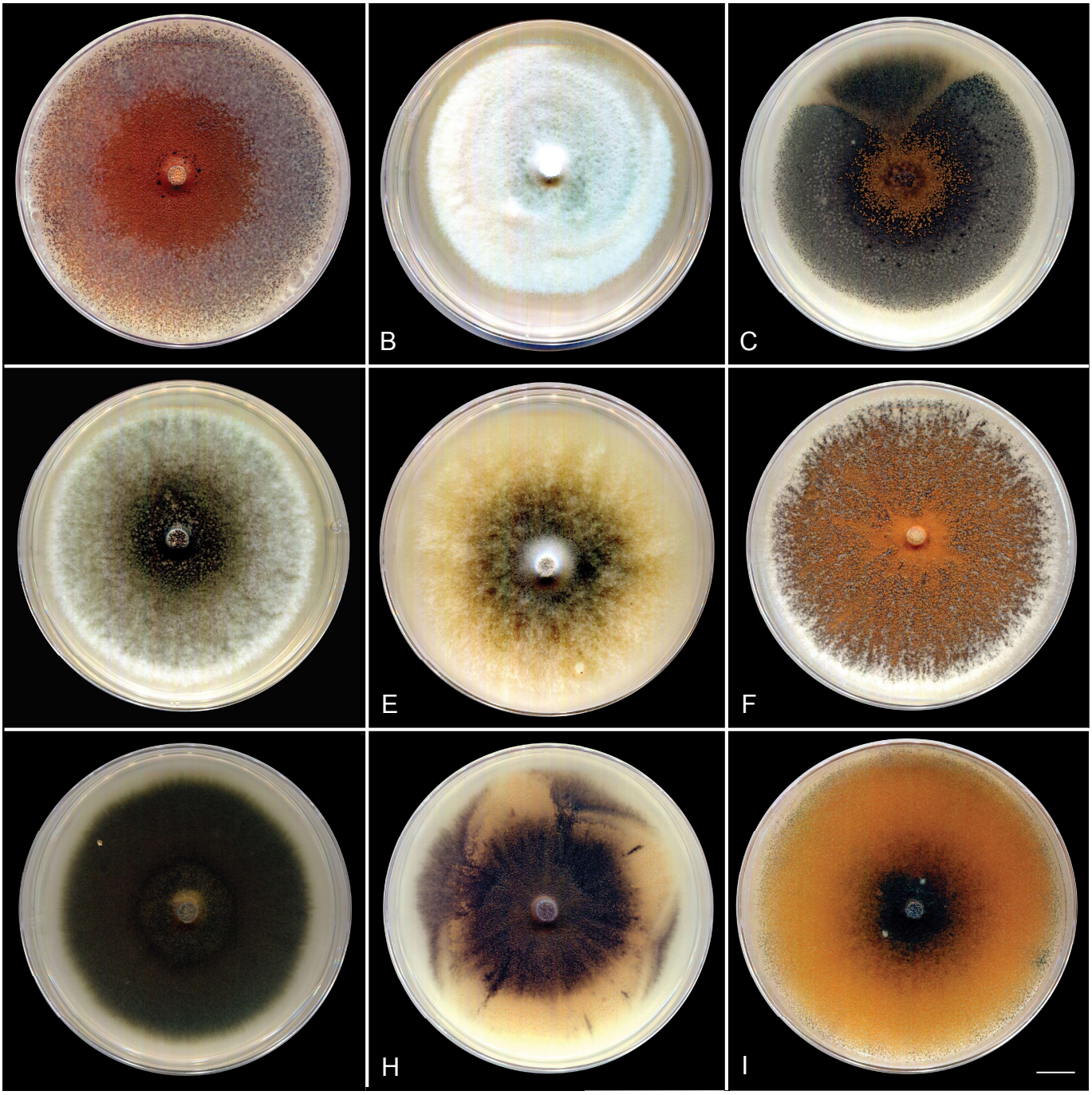


Fig. 1. Morphological types of *Colletotrichum* isolates from anthracnose lesions on banana fruits. Aerial view of cultures on PDA, 7d growth. A. Morphological type representative of Group 1, B-I. Group 2. Scale bar = 10 mm.

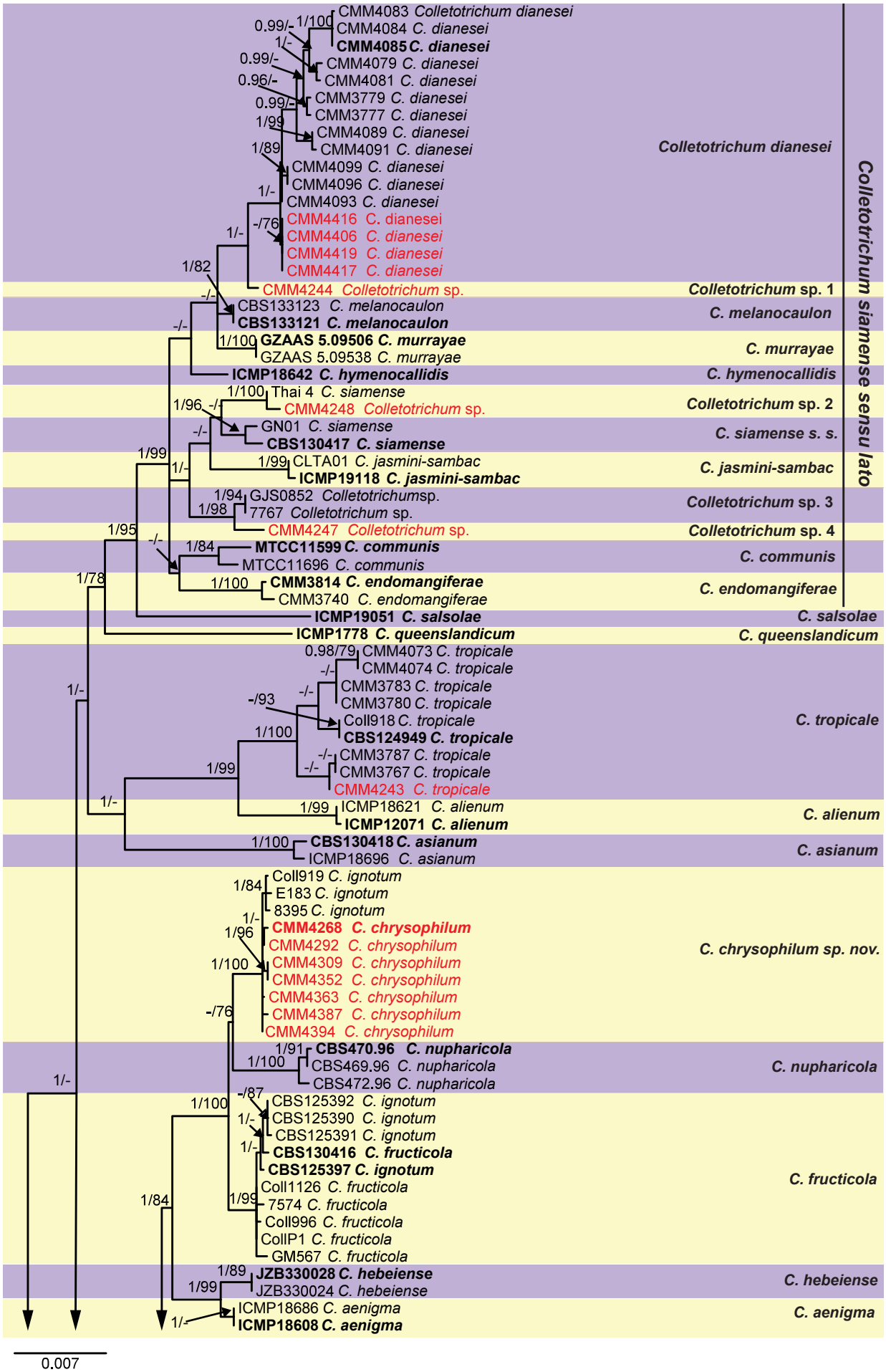


Fig. 2. . Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from a concatenated alignment of ACT, APN2, APN2/MAT-IGS, CAL, CHS-1, GAPDH, GAP2-IGS, GS, ITS and TUB2. Significant support for the Bayesian inference (≥0.95) and Maximum Likelihood (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are emphasized in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. theobromicola* and *C. grevilleae* were used as outgroup taxa The scale bar indicates the average number of substitutions per site.

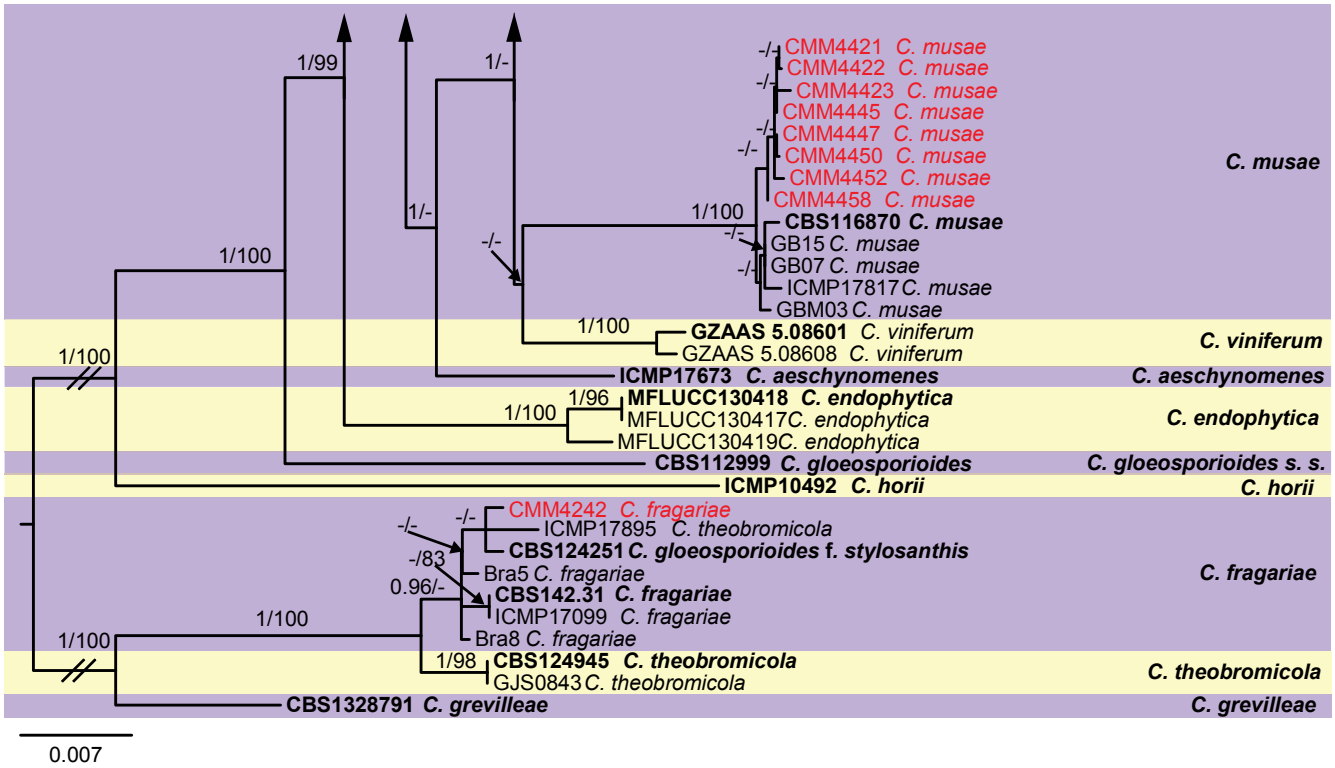

1/99
1/-
1/-
-/-
CMM4421 C. musae
CMM4422 C. musae
-/-
CMM4423 C. musae
CMM4445 C. musae
-/-
CMM4447 C. musae
-/-
CMM4450 C. musae
CMM4452 C. musae
CMM4458 C. musae
1/100
CBS116870 C. musae
-/-
GB15 C. musae
GB07 C. musae
-/-
ICMP17817 C. musae
GBM03 C. musae
C. musae
1/100
-/-
1/100
1/100
GZAAS 5.08601 C. viniferum
GZAAS 5.08608 C. viniferum
C. viniferum
1/100
ICMP17673 C. aeschynomenes
C. aeschynomenes
1/96
MFLUCC130418 C. endophytica
1/100
MFLUCC130417 C. endophytica
MFLUCC130419 C. endophytica
C. endophytica
CBS112999 C. gloeosporioides
C. gloeosporioides s. s.
ICMP10492 C. horii
C. horii
CMM4242 C. fragariae
-/-
-/-
ICMP17895 C. theobromicola
CBS124251 C. gloeosporioides f. stylosanthis
-/83
Bra5 C. fragariae
0.96/-
CBS142.31 C. fragariae
ICMP17099 C. fragariae
1/100
Bra8 C. fragariae
C. fragariae
1/100
1/98
CBS124945 C. theobromicola
GJS0843 C. theobromicola
C. theobromicola
CBS1328791 C. grevilleae
C. grevilleae
0.007

Fig. 2. (Ctd).

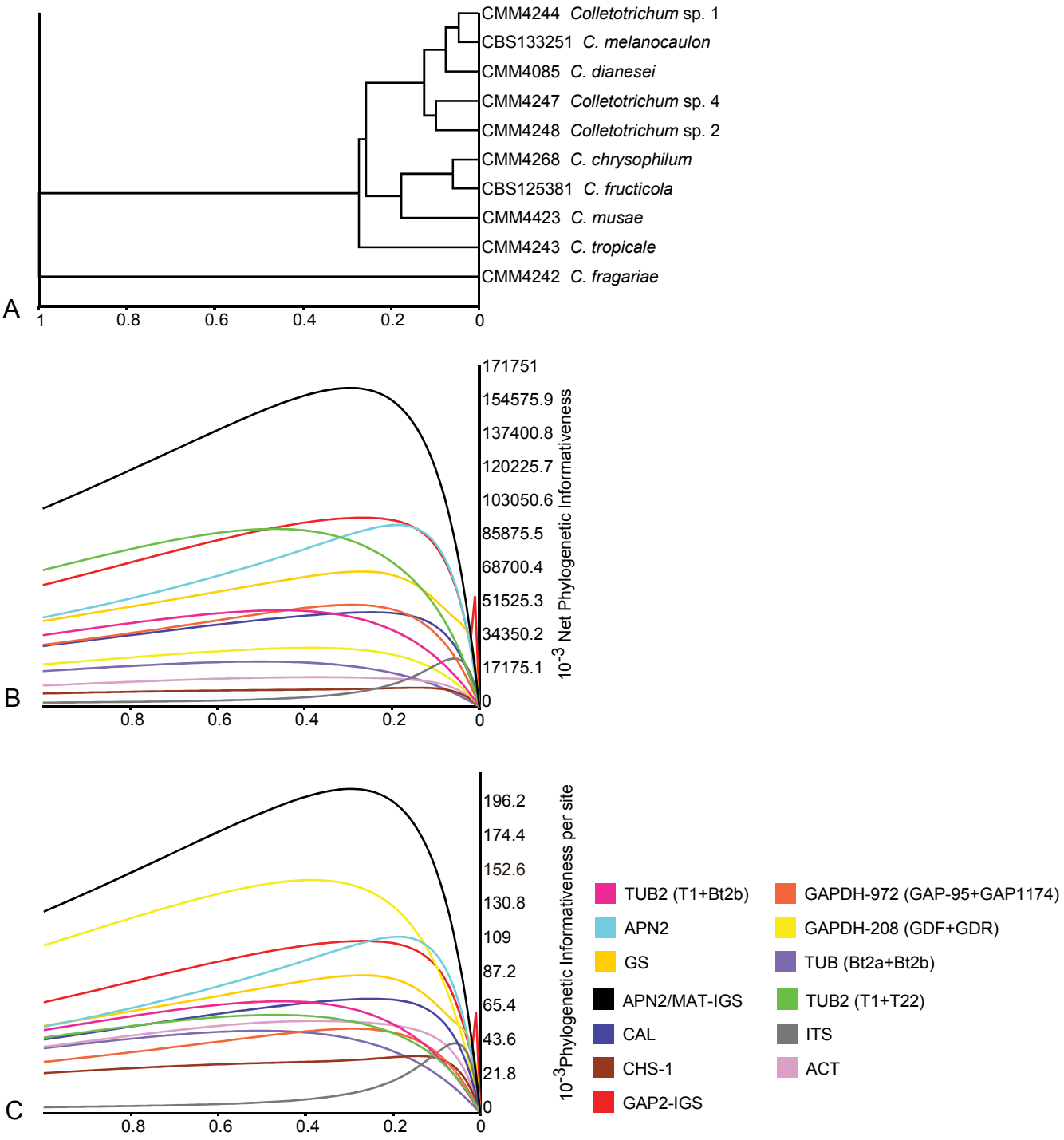


Fig. 3. Phylogenetic informativeness profiles of 13 amplicons within the *Colletotrichum gloeosporioides* species complex. A. Chronogram of the ML tree inferred from the concatenated data set of 10 loci (ACT, APN2, APN2/MAT-IGS, CAL, CHS-2, GAP2-IGS, GAPDH, GS and TUB2) and the corresponding net (B) and per site (C) phylogenetic informativeness profile for each amplicon. Values on the X-axes correspond to the relative timescale based on the root-to-tip distance of the Colletotrichum phylogeny.

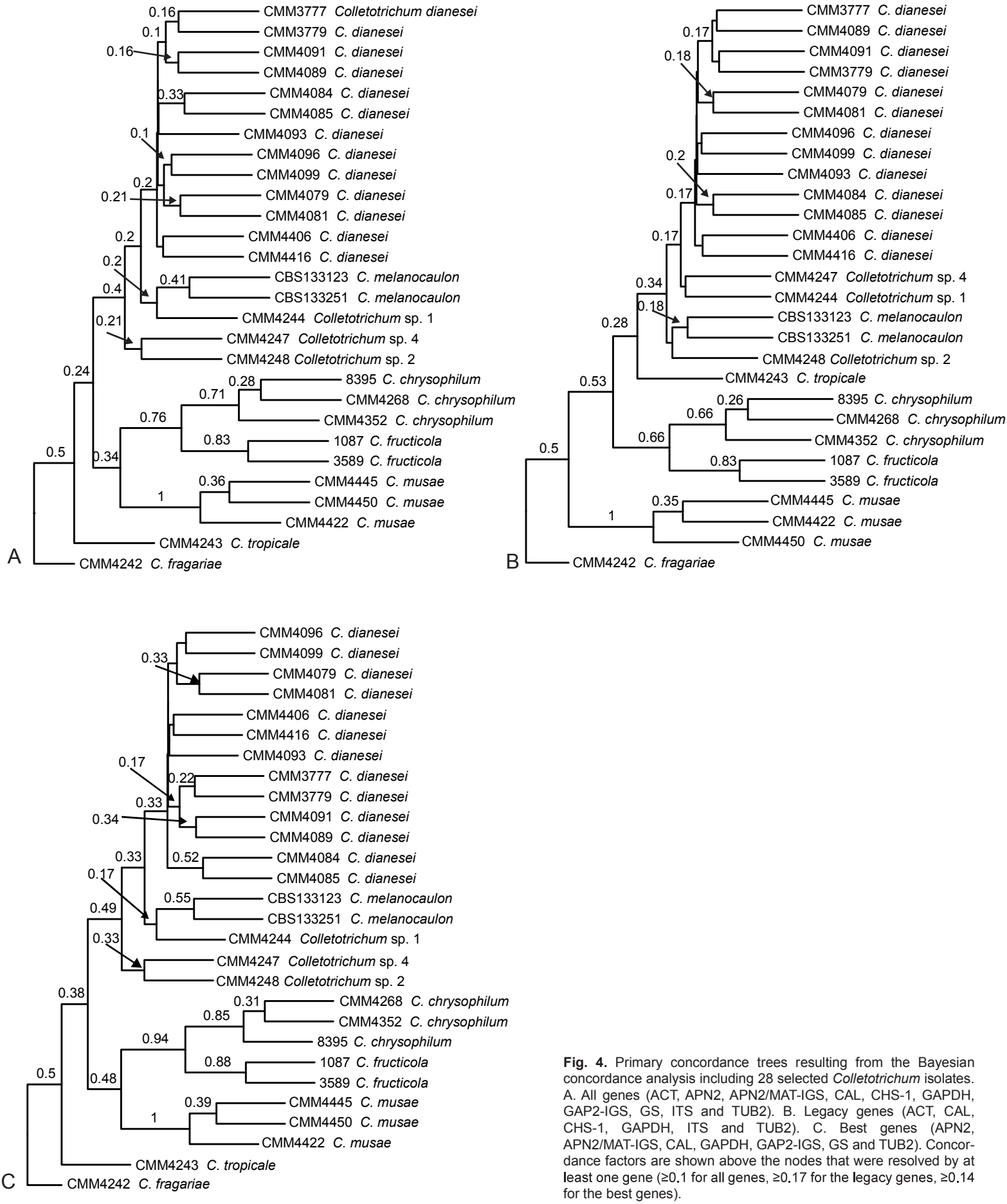


Fig. 4. Primary concordance trees resulting from the Bayesian concordance analysis including 28 selected *Colletotrichum* isolates. A. All genes (ACT, APN2, APN2/MAT-IGS, CAL, CHS-1, GAPDH, GAP2-IGS, GS, ITS and TUB2). B. Legacy genes (ACT, CAL, CHS-1, GAPDH, ITS and TUB2). C. Best genes (APN2, APN2/MAT-IGS, CAL, GAPDH, GAP2-IGS, GS and TUB2). Concordance factors are shown above the nodes that were resolved by at least one gene (≥0.1 for all genes, ≥0.17 for the legacy genes, ≥0.14 for the best genes).

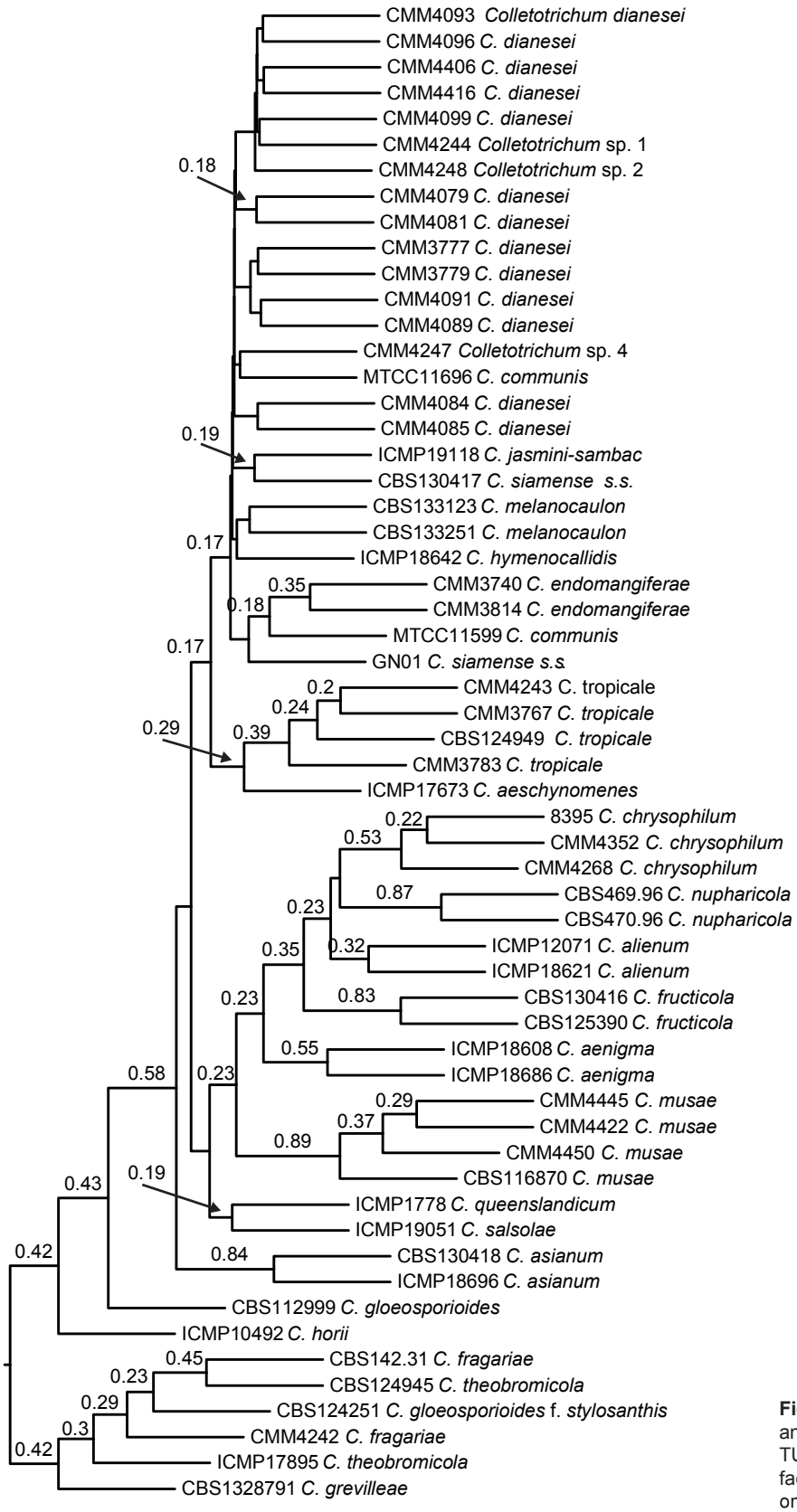


Fig. 5. Primary concordance tree from the Bayesian concordance analysis of the legacy genes (ACT, CAL, CHS-1, GAPDH, ITS and TUB2) from 58 selected *Colletotrichum* isolates. Concordance factors are shown above the nodes that were resolved by at least one gene (≥0.17).

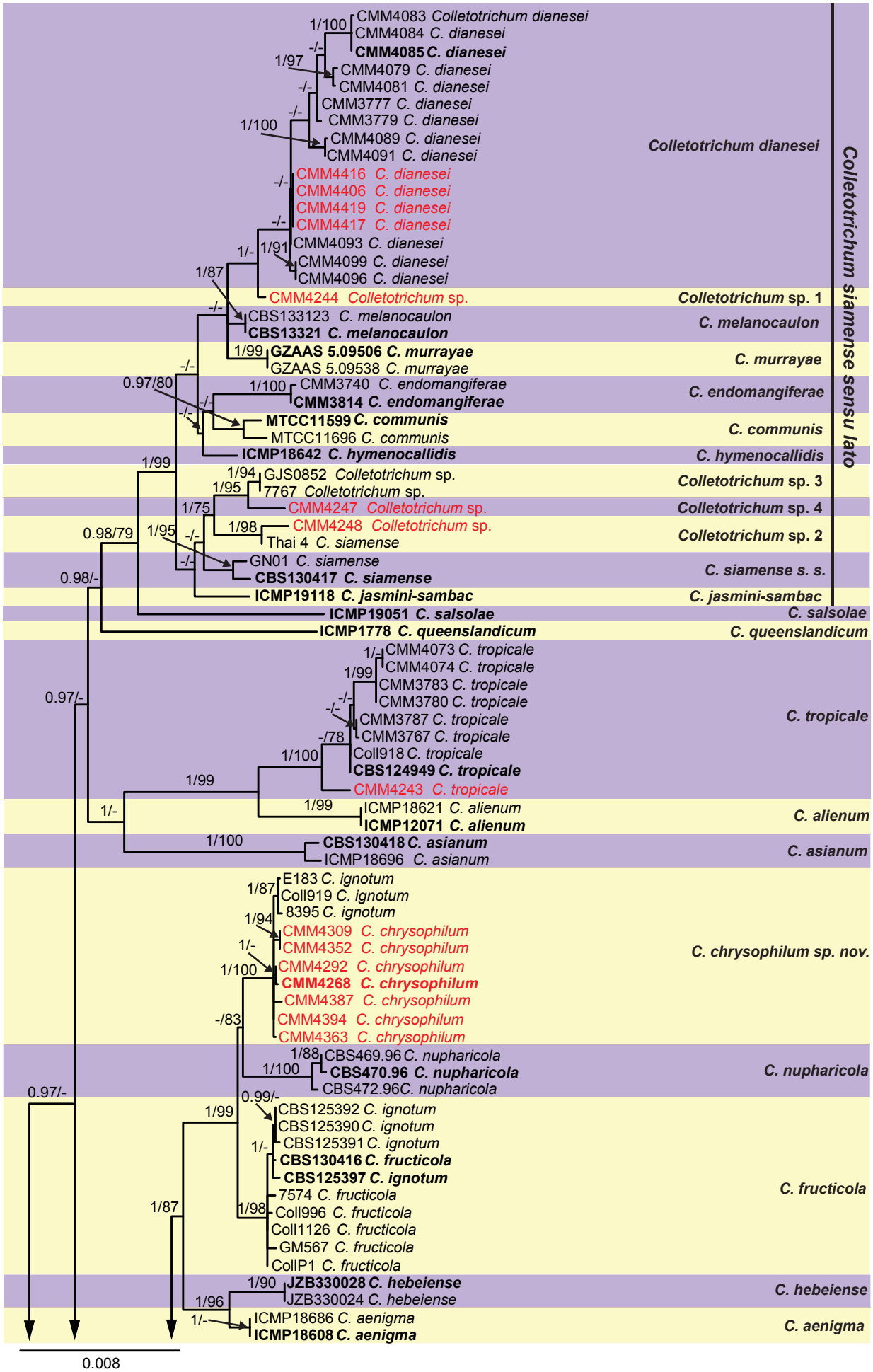
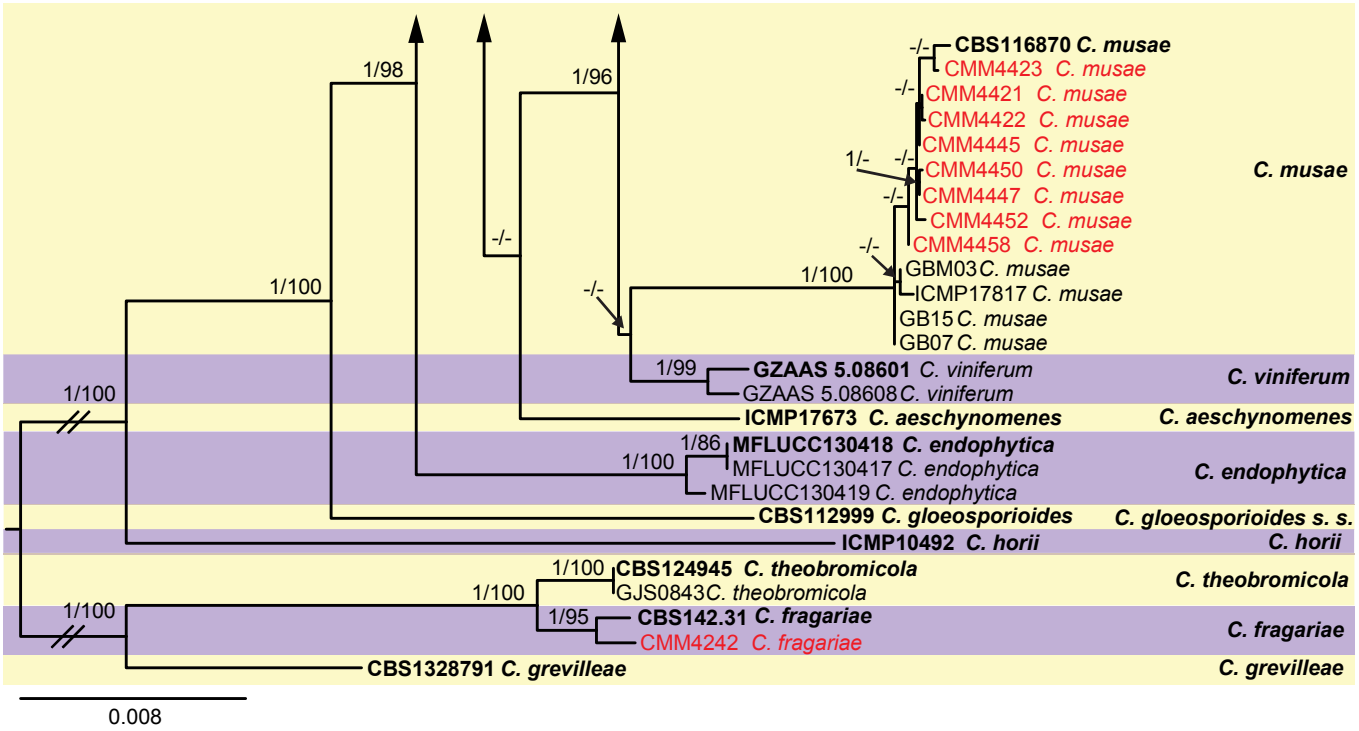


Fig. 6. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from a concatenated alignment of APN2, APN2/MAT-IGS, CAL, GAPDH, GAP2-IGS, GS and TUB2. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. theobromicola* and *C. grevilleae* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.


CBS116870 C. musae
CMM4423 C. musae
CMM4421 C. musae
CMM4422 C. musae
CMM4445 C. musae
CMM4450 C. musae
CMM4447 C. musae
CMM4452 C. musae
CMM4458 C. musae
GBM03 C. musae
ICMP17817 C. musae
GB15 C. musae
GB07 C. musae
C. musae
GZAAS 5.08601 C. viniferum
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ICMP17673 C. aeschynomenes
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C. endophytica
CBS112999 C. gloeosporioides
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GJS0843 C. theobromicola
C. theobromicola
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CMM4242 C. fragariae
C. fragariae
CBS1328791 C. grevilleae
C. grevilleae
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0.008

Fig. 2. (Ctd).

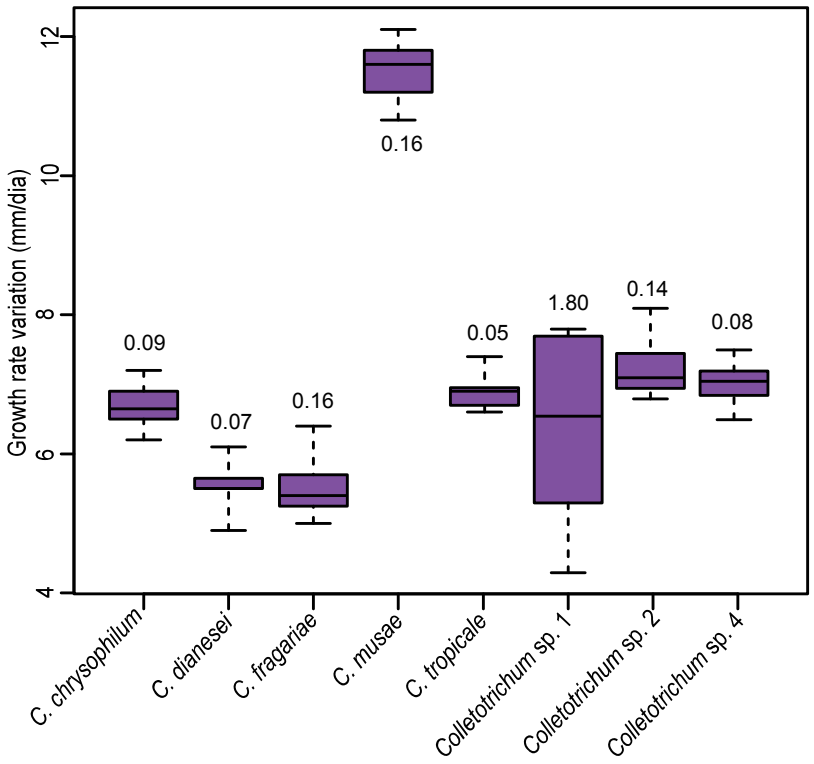

Fig. 7. Box plots representing the growth rate of *Colletotrichum s*pecies grown on PDA at 25 °C, under continuous light. Values above the boxes indicate the variance in growth rate.

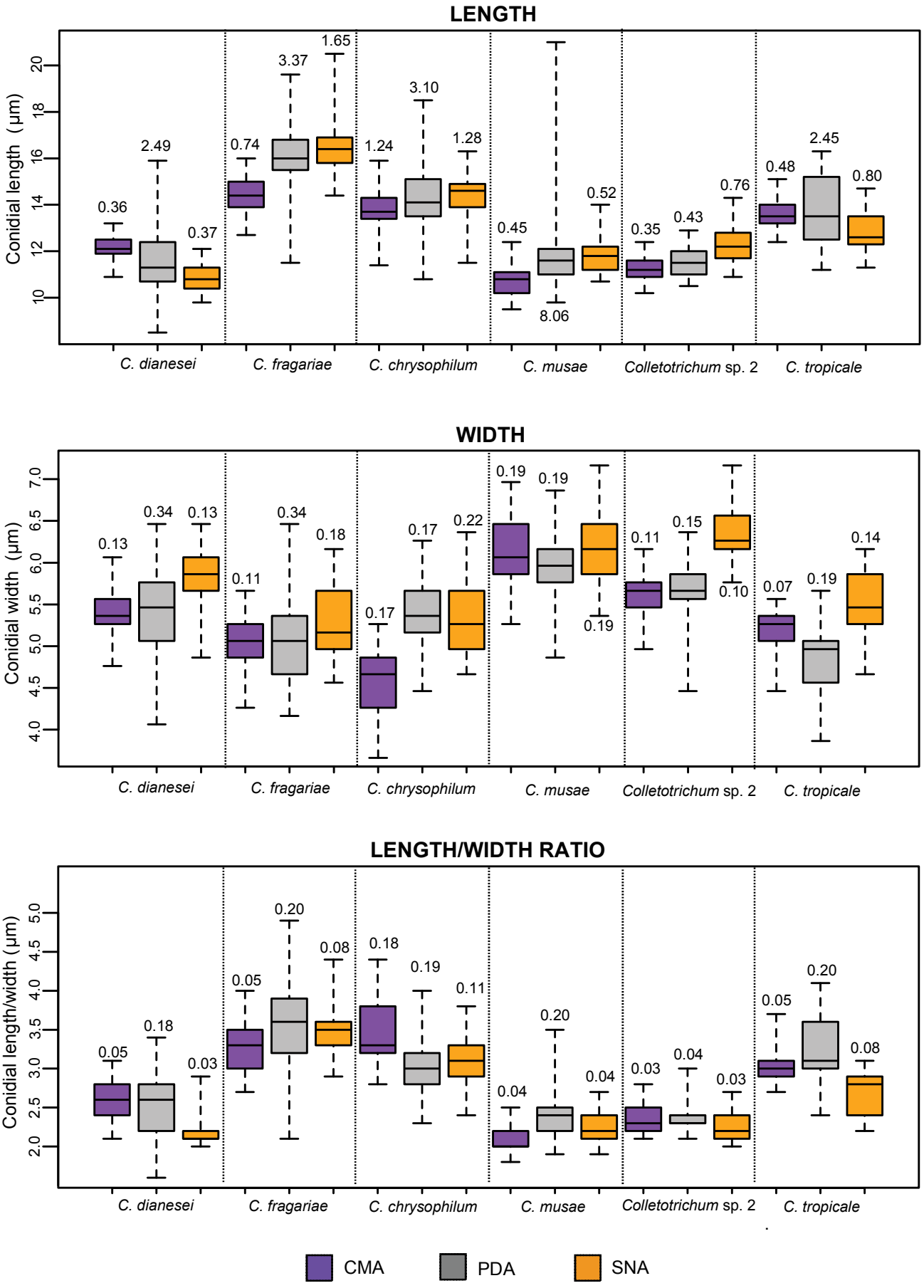

Fig. 8. Box plots representing the effect of different culture media on the conidial length, width and length/width ratio of *Colletotrichum* species. Values above or below the box indicate the variance.

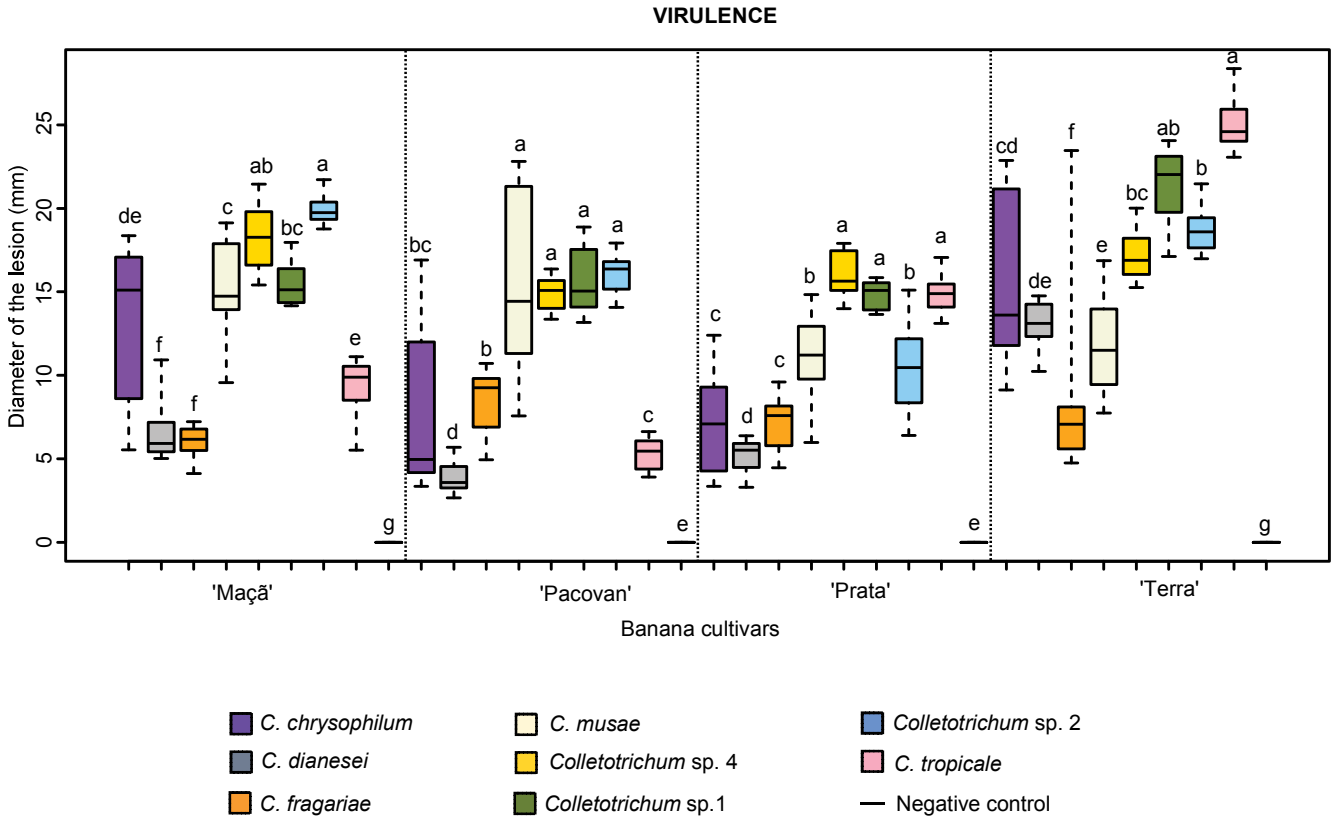

Fig. 9. Box plots representing the lesion length (mm) caused by Colletotrichum species on four cultivars of banana fruit four days after inoculation. Fruits inoculated with distilled water were used as the negative control. Data were previously transformed to log(x+1) for the statistical analysis. Boxes with same letter (above) do not differ significantly according to Fisher's LSD test (P≤0.05).

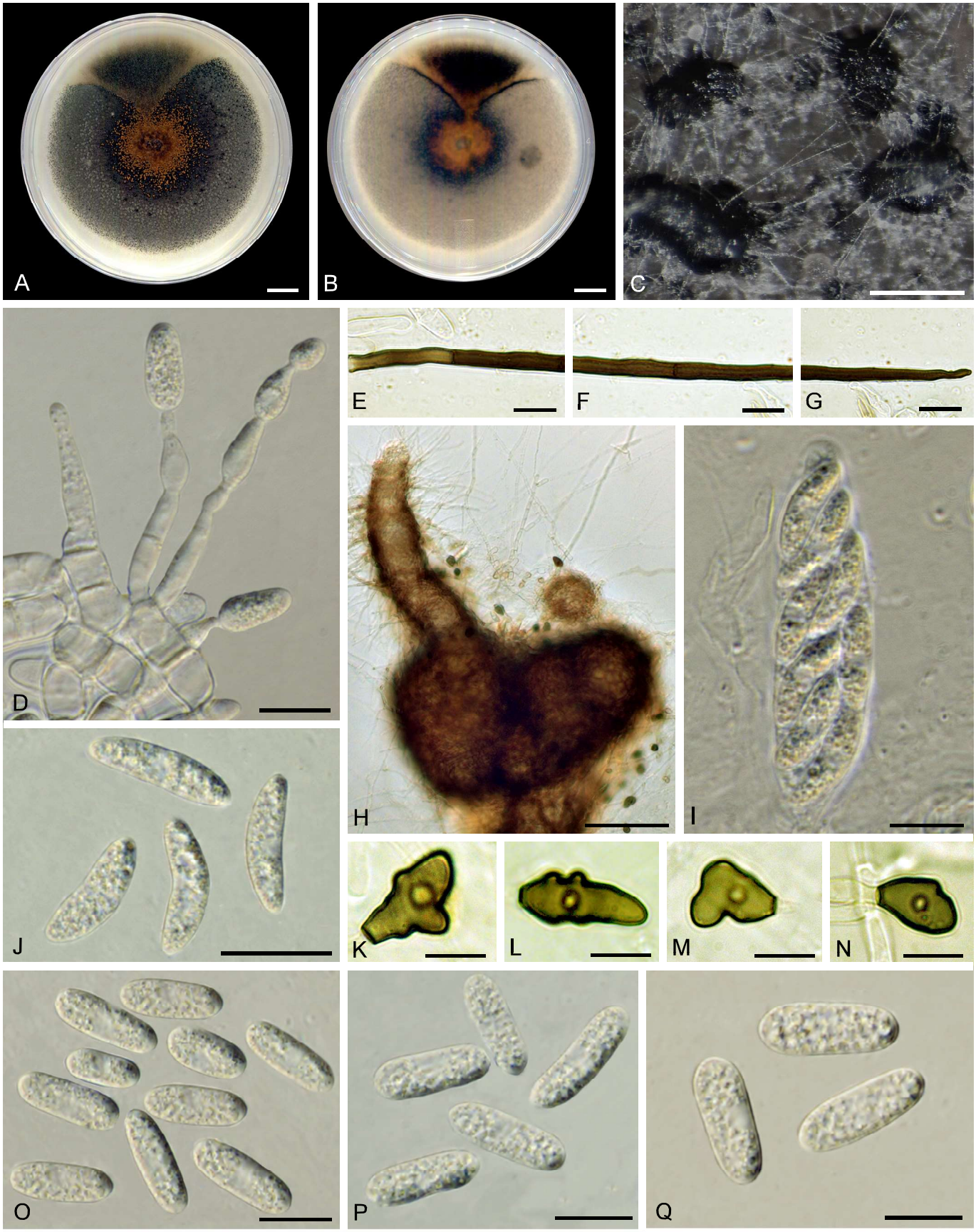

Fig 10. *Colletotrichum chrysophilum* CMM4268 – ex-holotype culture. A—B. Cultures on PDA, 7 d growth from CMA cultures, from above (A) and below (B). C, H. Perithecia. D. Conidiophores. E—G. Setae. I. Asci and ascospores. J. Ascospores. K—N. Appressoria. O—Q. Conidia. D—H, K—O. from CMA. P. from PDA. C, I, J, Q. from SNA. Scale bars: A, B = 10 mm; C,H = 100 µm; D—G, I, J, O—P = 10 µm; K—N = 5 µm.

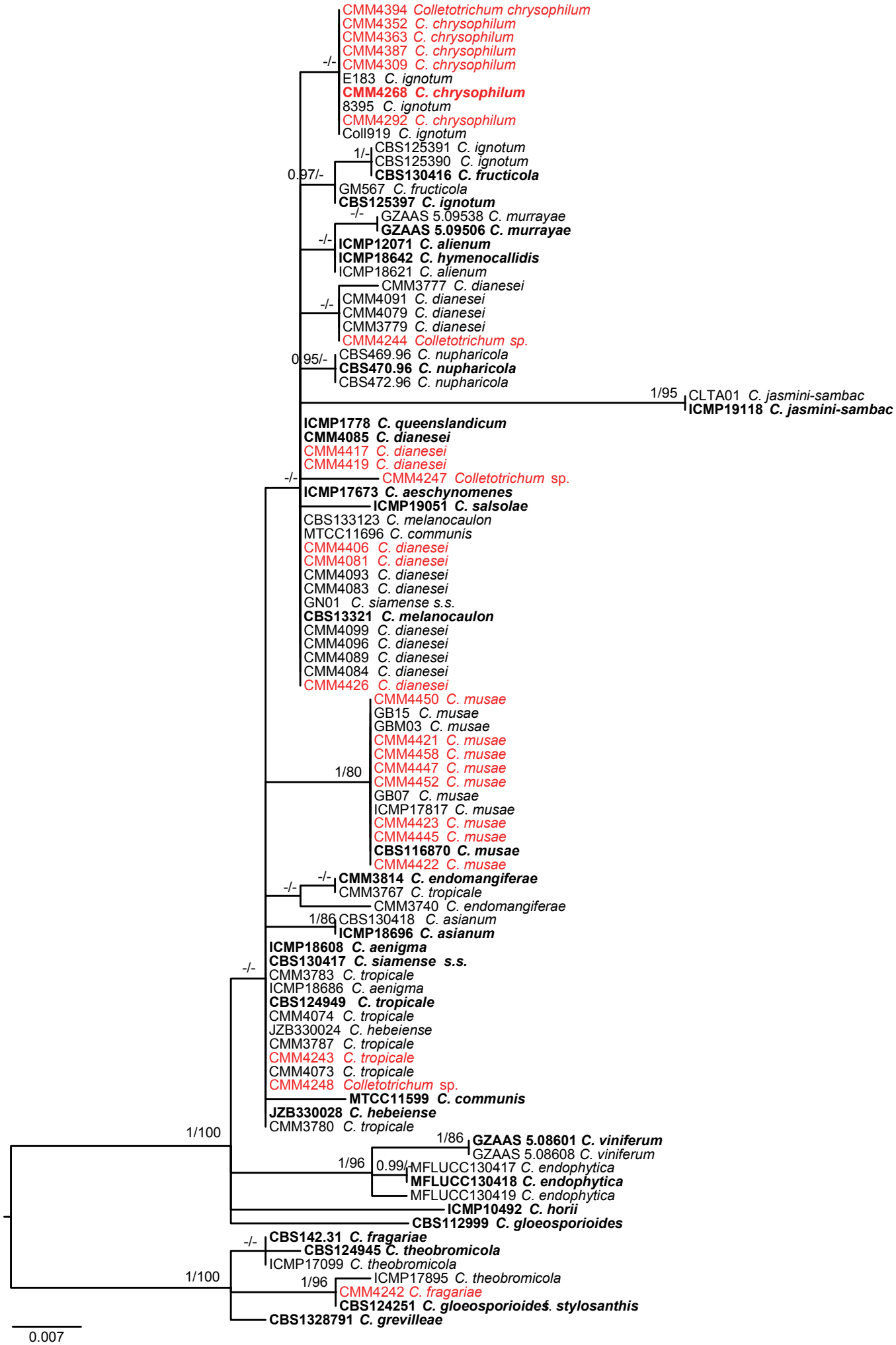

Fig. S1. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the ACT alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae*, *C. gloeosporioides* f. *stylosanthis, C. grevillae* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

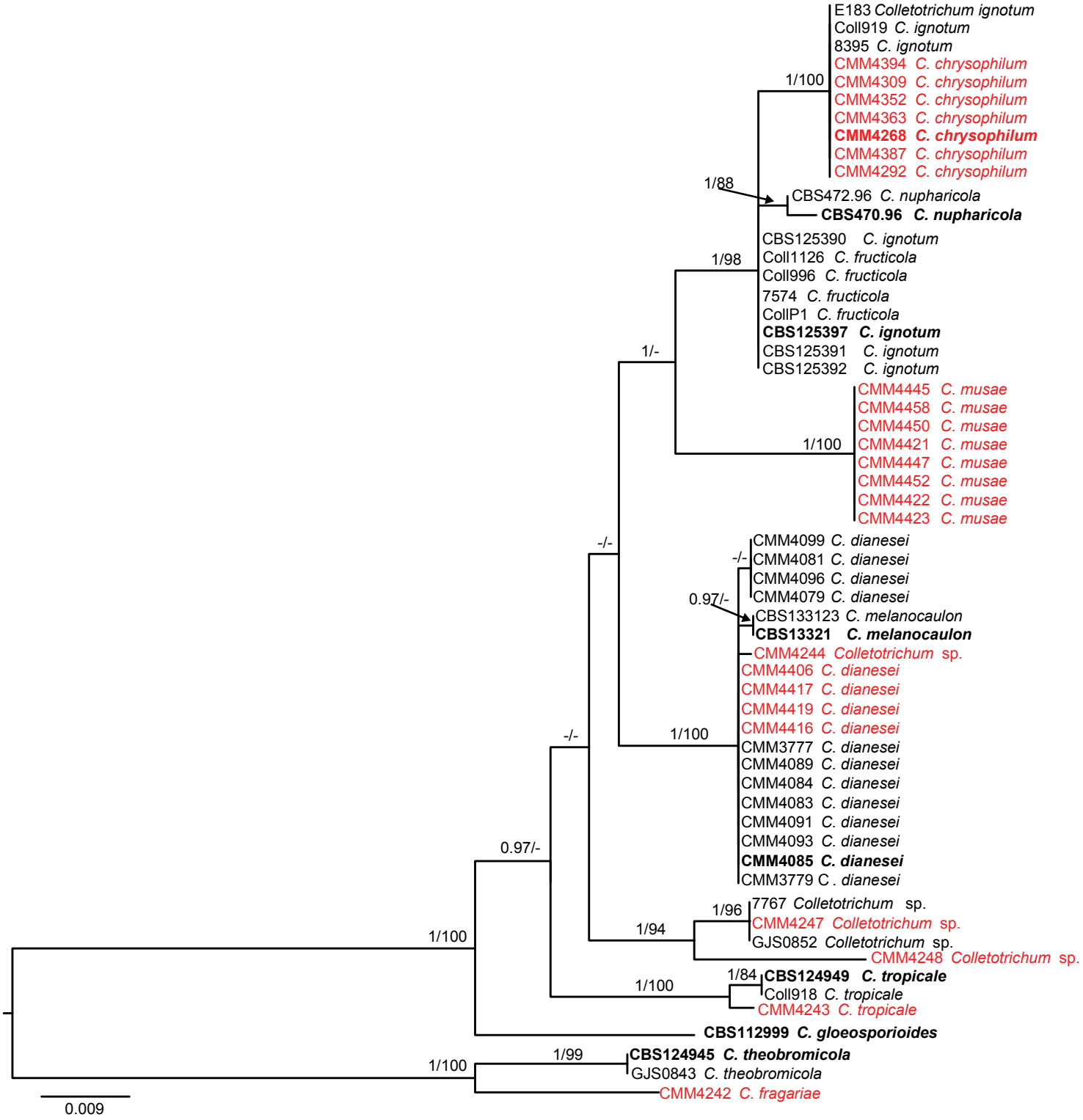

Fig. S2. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the APN2 alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

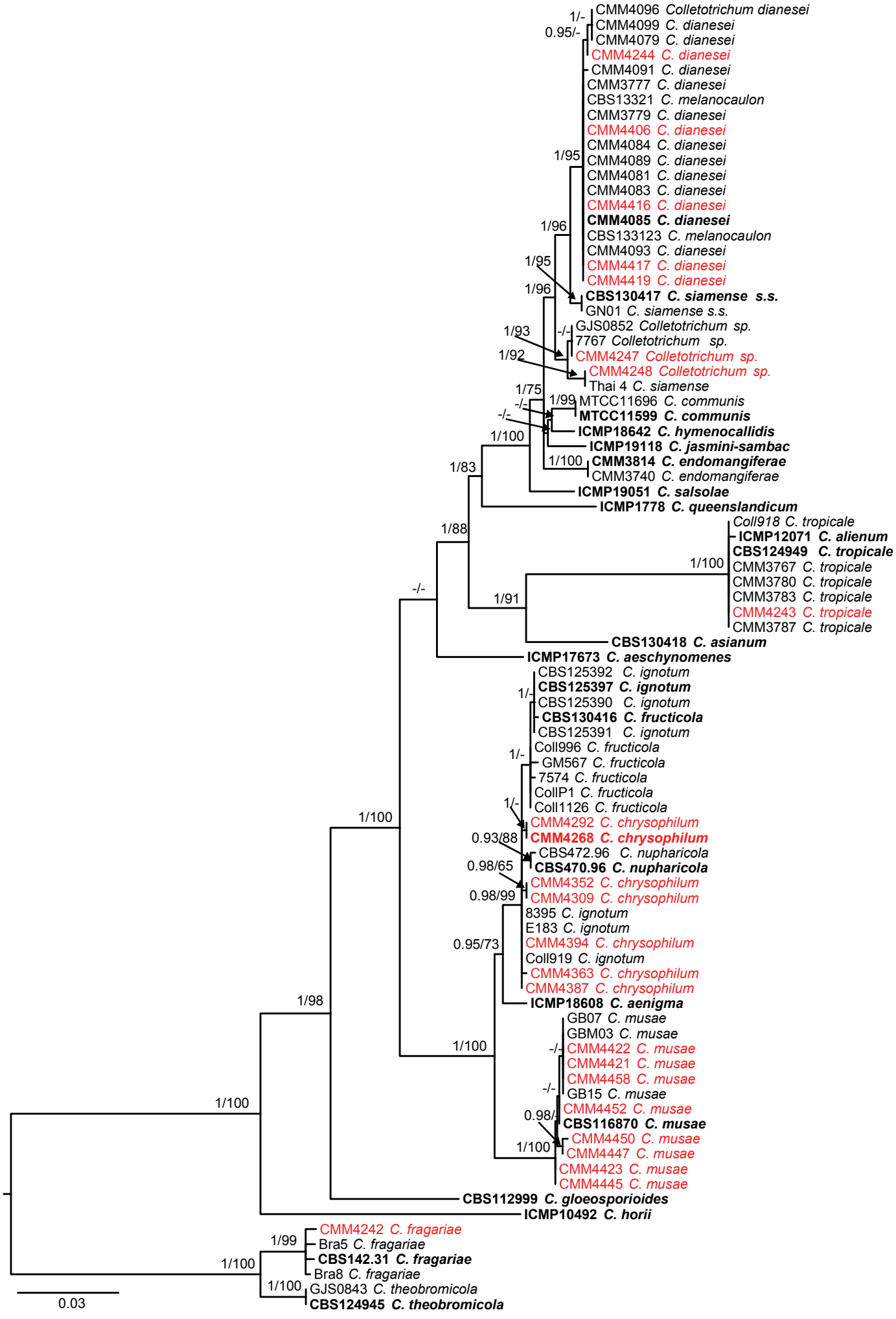

Fig. S3. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the APN2/MAT-IGS alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

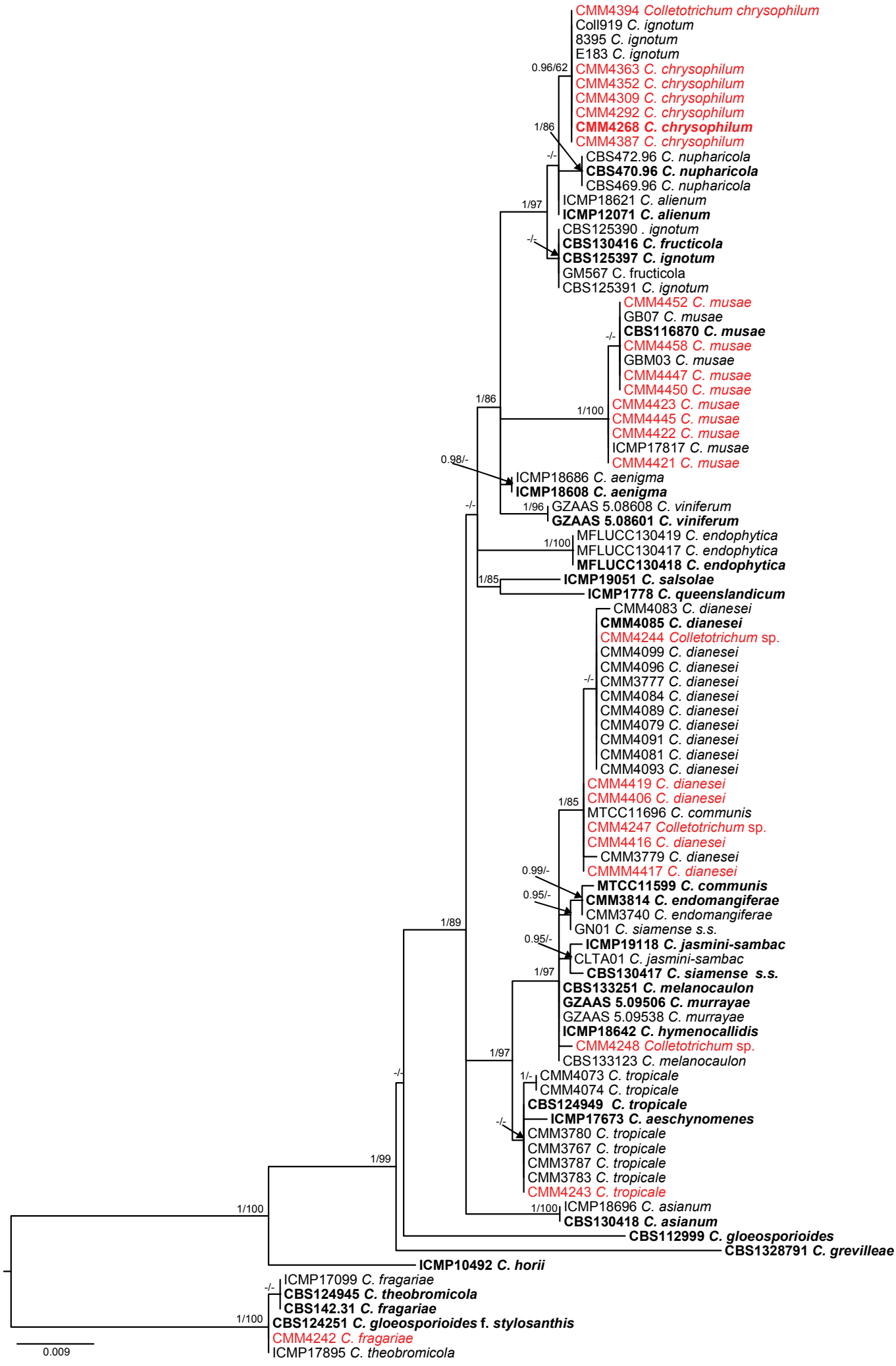

Fig. S4. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the CAL alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. gloeosporioides* f. *stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

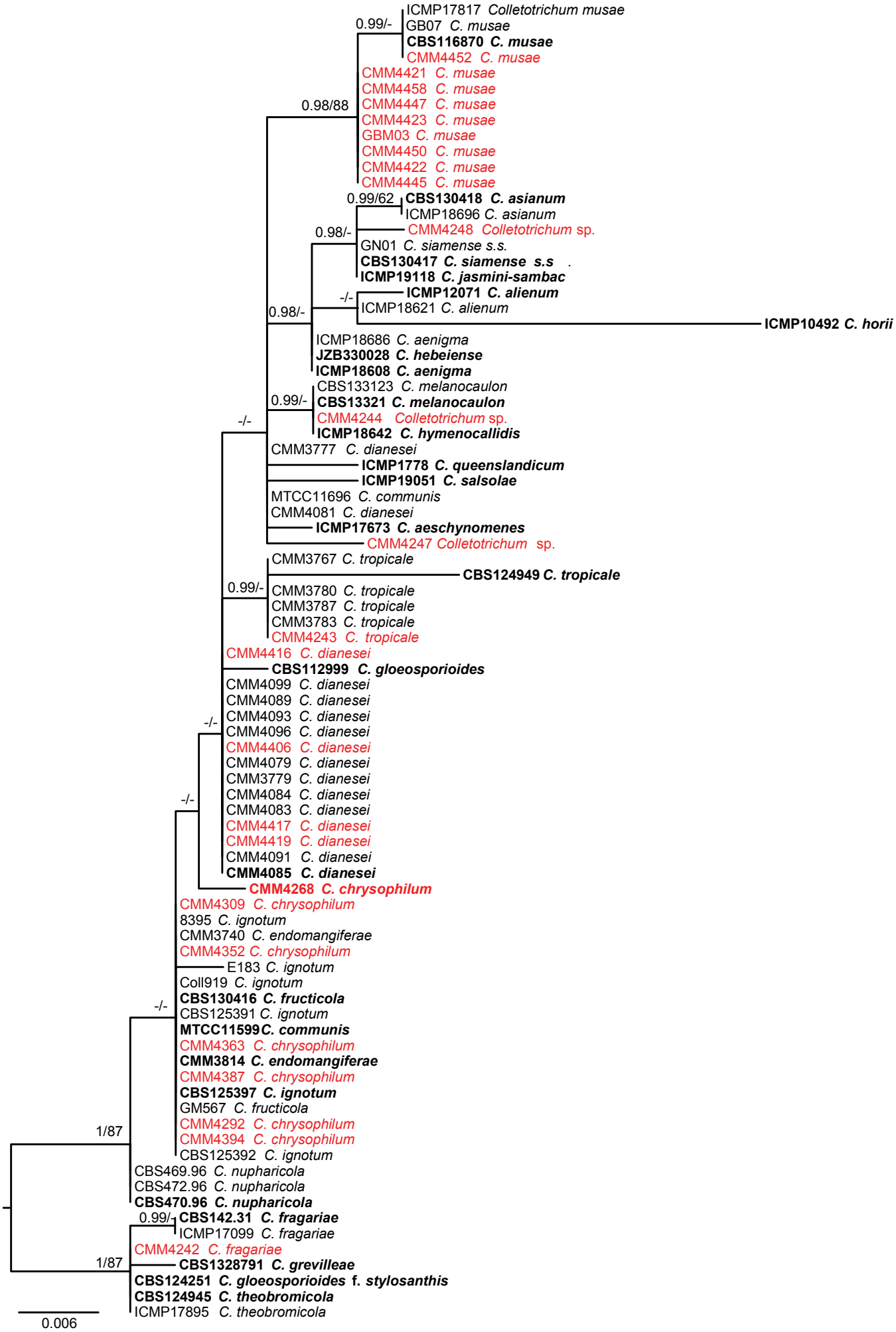

Fig. S5. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the CHS-1 alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. grevilleae, C. gloeosporioides f. stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

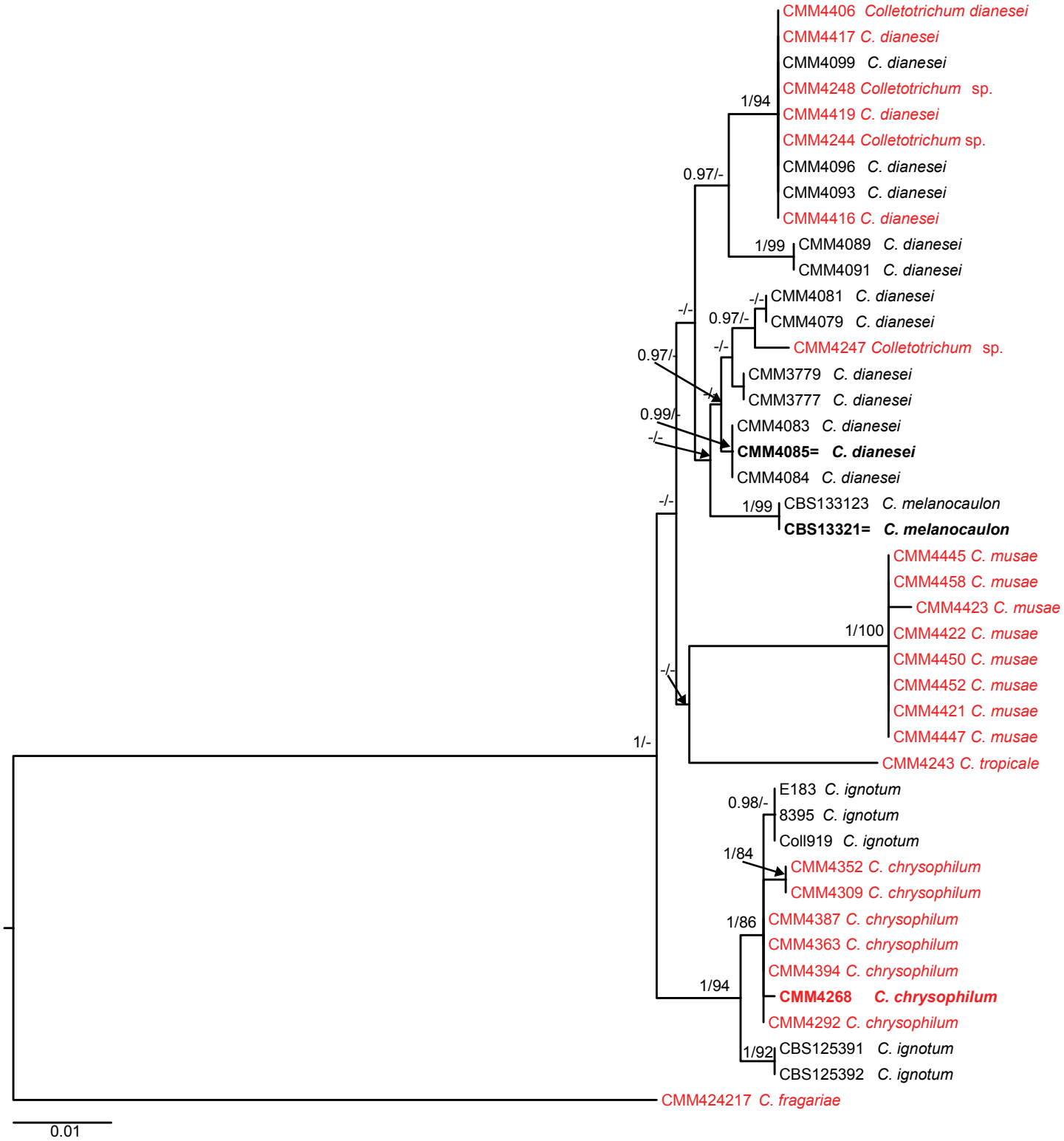

Fig. S6. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the GAP2-IGS alignment. Significant support for the Bayesian inference (≥ 0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae* was used as outgroup taxon. The scale bar indicates the average number of substitutions per site.

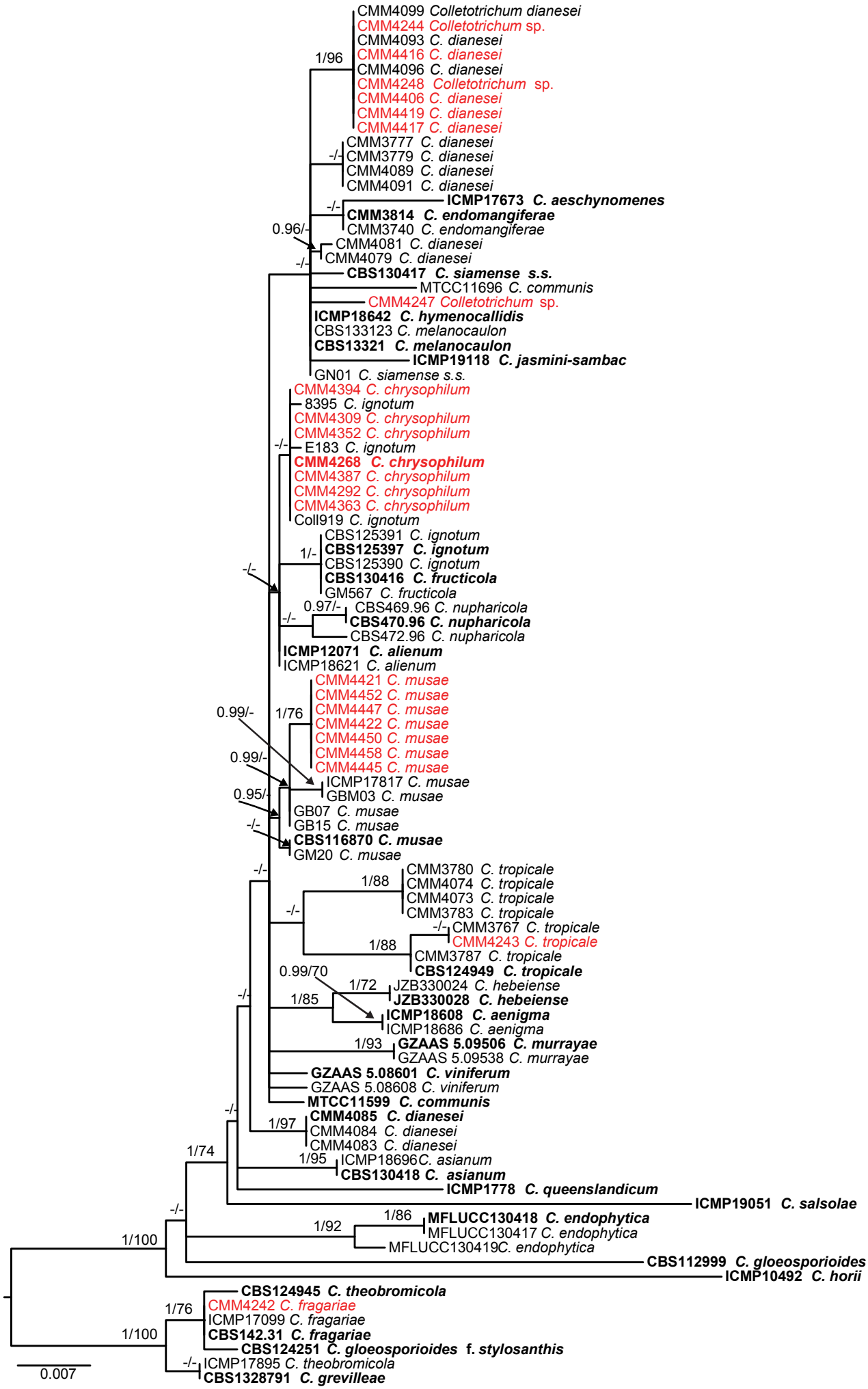

Fig. S7. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the GAPDH alignment. Significant support for the Bayesian inference (≥ 0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. grevilleae, C. gloeosporioides f. stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

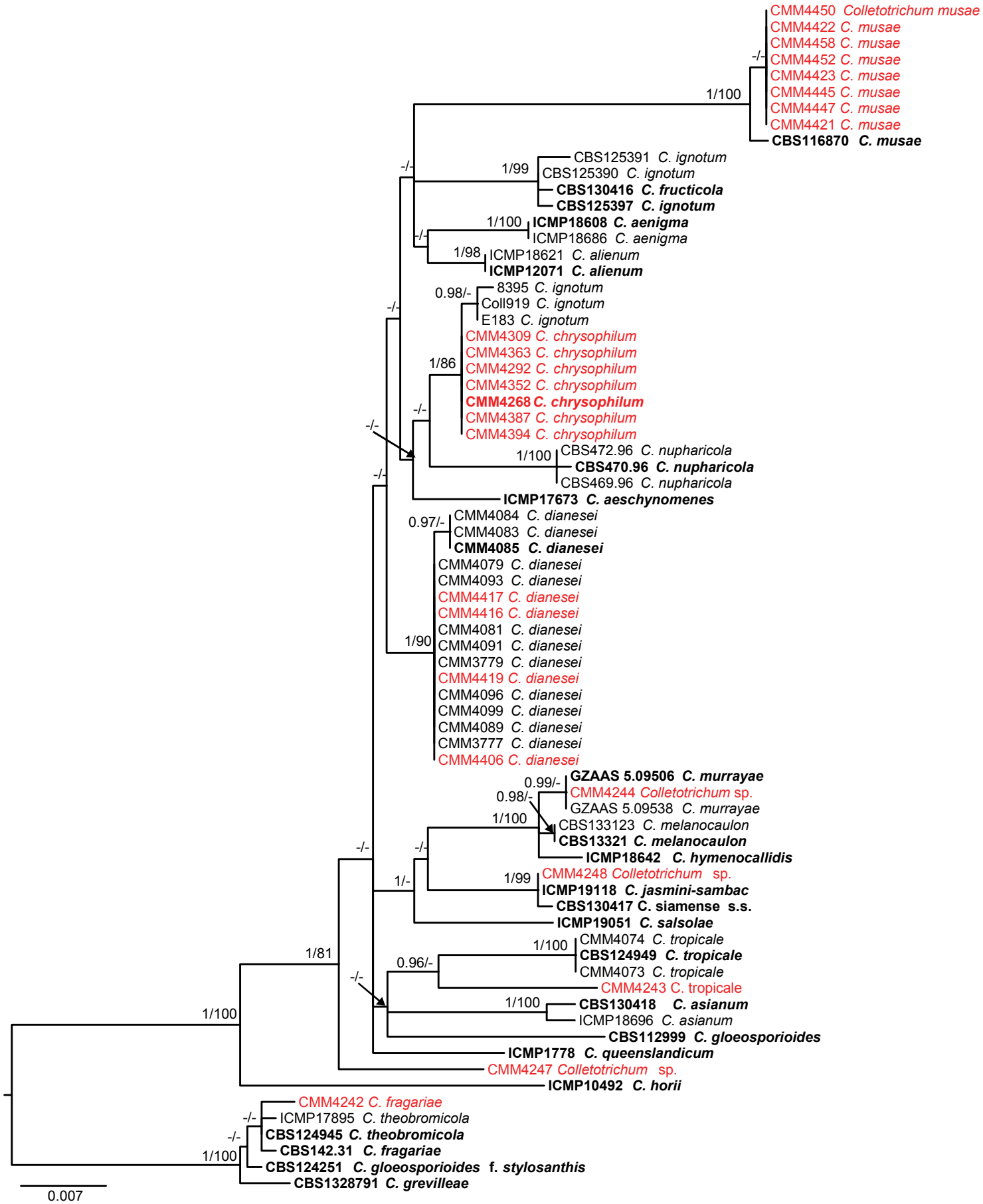

Fig. S8. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the GS alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. grevilleae, C. gloeosporioides f. stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

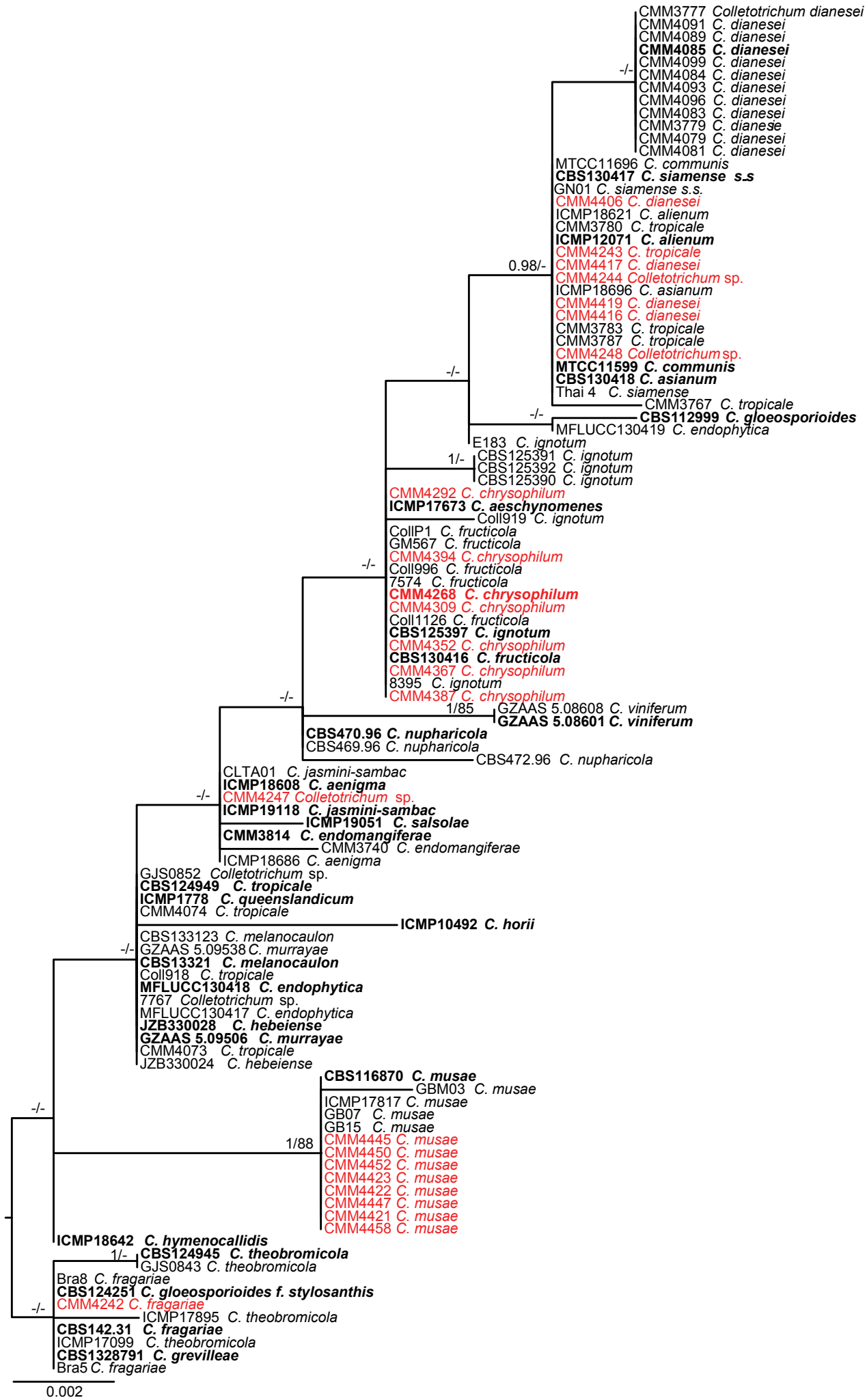

Fig. S9. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the ITS alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. grevilleae, C. gloeosporioides f. stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

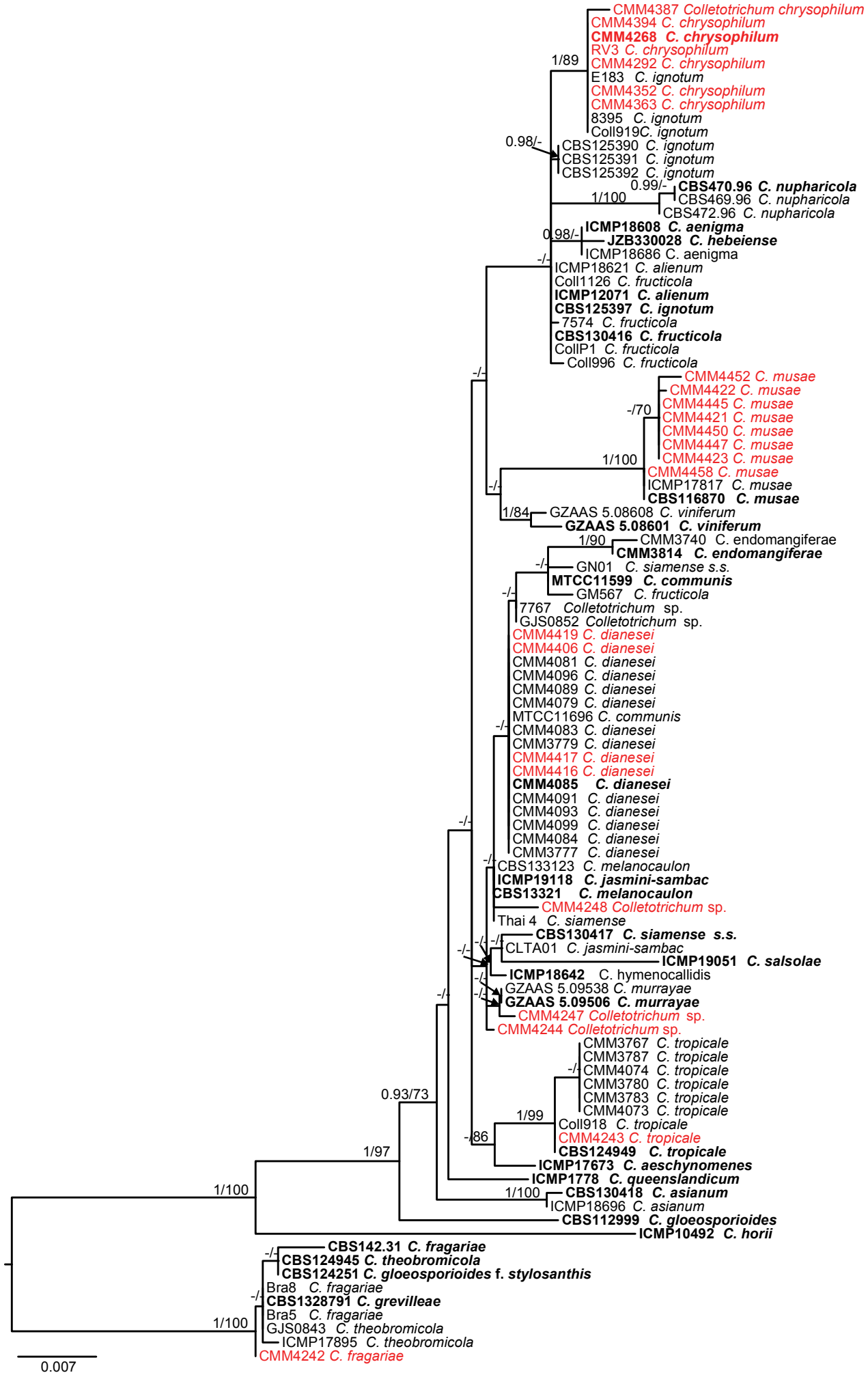

Fig. S10. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from the TUB2 alignment. Significant support for the Bayesian inference (≥0.95) and ML (≥70) are shown above the nodes (PP/ML-BS). "-" indicates no-significant support or absence of the node. Ex-types are in bold and include the taxonomic name as originally described. Isolates from banana are highlighted in red. *Colletotrichum fragariae, C. grevilleae, C. gloeosporioides f. stylosanthis* and *C. theobromicola* were used as outgroup taxa. The scale bar indicates the average number of substitutions per site.es per site.

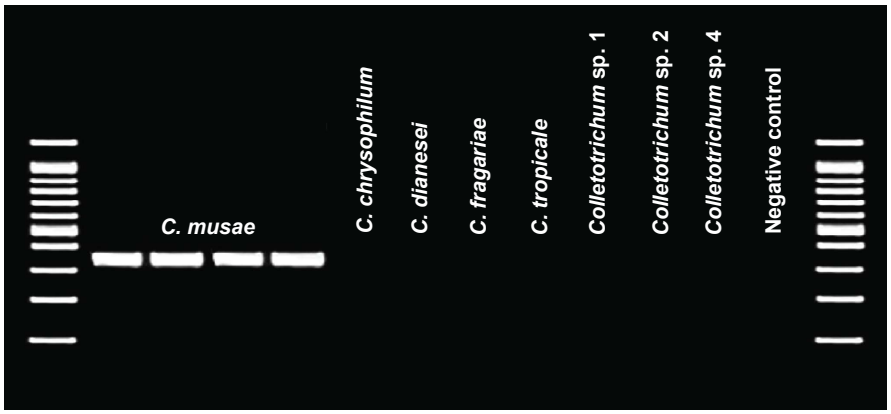

Fig. S11. Agarose gel (2%) showing an amplicon of approximately 350 bp for the *C. musae* isolates using the *C. musae* species-specific primers generated in the present study. First and last lane are 100 bp ladder (Promega®).

Table 1. Strains of *Colletotrichum* spp. studied, with collection details and GenBank accessions.

Species	Culture[1]	Host	Country	GenBank accession number[2]									
				ACT	APN2	APN2/MAT-IGS	CAL	CHS-1	GAP2-IGS	GAPDH	GS	ITS	TUB2
C. aenigma	ICMP 18608*	*Persea americana*	Israel	JX009443	-	KM360143	JX009683	JX009774	-	JX010044	JX010078	JX010244	JX010389
C. aenigma	ICMP 18686	*Pyrus pyrifolia*	Japan	JX009519	-	-	JX009684	JX009789	-	JX009913	JX010079	JX010243	JX010390
C. aeschynomenes	ICMP 17673, ATCC 201874*	*Aeschynomene virginica*	USA	JX009483	-	KM360145	JX009721	JX009799	-	JX009930	JX010081	JX010176	JX010392
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	JX009572	-	KC888927	JX009654	JX009882	-	JX010028	JX010101	JX010251	JX010411
C. alienum	ICMP 18621	*Persea americana*	New Zealand	JX009552	-	-	JX009657	JX009755	-	JX009959	JX010075	JX010246	JX010386
C. asianum	ICMP 18580*, B26CBS 130418	*Coffea arabica*	Thailand	JX009584	-	FR718814	FJ917506	JX009867	-	JX010053	JX010096	FJ972612	JX010406
C. asianum	IMI 313839, ICMP 18696	*Mangifera indica*	Australia	JX009576	-	-	JX009723	JX009753	-	JX009915	JX010073	JX010192	JX010384
C. chrysophilum	CMM 4268*, URM 7362	*Musa* sp.	Brazil	**KX093982**	**KX094018**	**KX094325**	**KX094063**	**KX094083**	**KX094125**	**KX094183**	**KX094204**	**KX094252**	**KX094285**
C. chrysophilum	CMM 4292	*Musa* sp.	Brazil	**KX093981**	**KX094015**	**KX094324**	**KX094062**	**KX094079**	**KX094131**	**KX094182**	**KX094203**	**KX094248**	**KX094284**
C. chrysophilum	CMM 4309	*Musa* sp.	Brazil	**KX093984**	**KX094021**	**KX094327**	**KX094065**	**KX094086**	**KX094124**	**KX094185**	**KX094206**	**KX094255**	**KX094287**
C. chrysophilum	CMM 4352	*Musa* sp.	Brazil	**KX093983**	**KX094020**	**KX094326**	**KX094064**	**KX094085**	**KX094123**	**KX094184**	**KX094205**	**KX094254**	**KX094286**
C. chrysophilum	CMM 4363	*Musa* sp.	Brazil	**KX093962**	**KX094007**	**KX094323**	**KX094060**	**KX094071**	**KX094130**	**KX094180**	**KX094201**	**KX094240**	**KX094283**
C. chrysophilum	CMM 4387	*Musa* sp.	Brazil	**KX093980**	**KX094013**	**KX094322**	**KX094061**	**KX094077**	**KX094132**	**KX094181**	**KX094202**	**KX094246**	**KX094281**
C. chrysophilum	CMM 4394	*Musa* sp.	Brazil	**KX093979**	**KX094006**	**KX094321**	**KX094059**	**KX094070**	**KX094129**	**KX094179**	**KX094200**	**KX094239**	**KX094282**
C. chrysophilum (*C. ignotum*)	8395	*Theobroma cacao*	Panama	**KX093976**	GU994415	GU994444	**KX094056**	**KX094106**	**KX094126**	**KX094176**	**KX094209**	GU994370	GU994473
C. chrysophilum (*C. ignotum*)	Coll919	*Terpsichore taxifolia*	Puerto Rico	**KX093977**	JX145265	JX145317	**KX094057**	**KX094107**	**KX094127**	**KX094177**	**KX094207**	JX145163	**KX094288**
C. chrysophilum (*C. ignotum*)	E183	*Genipa americana*	Panama	**KX093978**	GU994414	GU994443	**KX094058**	**KX094108**	**KX094128**	**KX094178**	**KX094208**	GU994367	GU994472
C. communis	GO01	*Citrus* sp.	India	KF451940	-	KC790720	KF451953	KF451988	-	KF452016	-	KC790977	KF452029
C. communis	NK24	*Mangifera indica*	India	JQ894546	-	JQ894582	KC790791	JQ894617	-	JQ894632	-	JQ894681	JQ894602

C. dianesei	CMM 3777	*Mangifera indica*	Brazil	KC702911	**KX093993**	**KX094302**	**KX094045**	**KX094092**	**KX094142**	**KX094162**	**KX094214**	KC702970	**KX094266**
C. dianesei	CMM 3779	*Mangifera indica*	Brazil	KC702912	**KX093994**	**KX094303**	**KX094043**	**KX094093**	**KX094143**	**KX094163**	**KX094215**	KC702971	**KX094267**
C. dianesei	CMM 4079	*Mangifera indica*	Brazil	KC533746	**KX093998**	**KX094307**	**KX094052**	**KX094097**	**KX094147**	**KX094167**	**KX094219**	KC329776	**KX094271**
C. dianesei	CMM 4081	*Mangifera indica*	Brazil	KC517304	**KX093999**	**KX094310**	**KX094053**	**KX094098**	**KX094148**	**KX094166**	**KX094220**	KC329790	**KX094272**
C. dianesei	CMM 4083	*Mangifera indica*	Brazil	KC517298	**KX093995**	**KX094304**	**KX094044**	**KX094094**	**KX094144**	**KX094156**	**KX094216**	KC329779	**KX094268**
C. dianesei	CMM 4084	*Mangifera indica*	Brazil	KC517307	**KX093996**	**KX094305**	**KX094050**	**KX094095**	**KX094145**	**KX094157**	**KX094217**	KC329811	**KX094269**
C. dianesei	CMM 4085*	*Mangifera indica*	Brazil	KC533740	**KX093997**	**KX094306**	**KX094051**	**KX094096**	**KX094146**	**KX094158**	**KX094218**	KC329813	**KX094270**
C. dianesei	CMM 4089	*Mangifera indica*	Brazil	KC517302	**KX094000**	**KX094311**	**KX094054**	**KX094099**	**KX094149**	**KX094164**	**KX094221**	KC329783	**KX094273**
C. dianesei	CMM 4091	*Mangifera indica*	Brazil	KC533737	**KX094001**	**KX094313**	**KX094046**	**KX094100**	**KX094150**	**KX094165**	**KX094222**	KC329804	**KX094274**
C. dianesei	CMM 4093	*Mangifera indica*	Brazil	KC533734	**KX094002**	**KX094312**	**KX094047**	**KX094101**	**KX094151**	**KX094159**	**KX094223**	KC329808	**KX094275**
C. dianesei	CMM 4096	*Mangifera indica*	Brazil	KC533733	**KX094003**	**KX094308**	**KX094048**	**KX094102**	**KX094152**	**KX094160**	**KX094224**	KC329806	**KX094276**
C. dianesei	CMM 4099	*Mangifera indica*	Brazil	KC517303	**KX094004**	**KX094309**	**KX094049**	**KX094103**	**KX094153**	**KX094161**	**KX094225**	KC329784	**KX094277**
C. dianesei	CMM 4406	*Musa sp.*	Brazil	**KX093989**	**KX094023**	**KX094319**	**KX094040**	**KX094088**	**KX094138**	**KX094169**	**KX094211**	**KX094257**	**KX094263**
C. dianesei	CMM 4416	*Musa sp.*	Brazil	**KX093990**	**KX094024**	**KX094317**	**KX094041**	**KX094089**	**KX094139**	**KX094170**	**KX094212**	**KX094258**	**KX094264**
C. dianesei	CMM 4417	*Musa sp.*	Brazil	**KX093988**	**KX094022**	**KX094318**	**KX094039**	**KX094087**	**KX094137**	**KX094168**	**KX094210**	**KX094256**	**KX094262**
C. dianesei	CMM 4419	*Musa sp.*	Brazil	**KX093991**	**KX094026**	**KX094316**	**KX094042**	**KX094091**	**KX094140**	**KX094171**	**KX094213**	**KX094260**	**KX094265**
C. endomangiferae	CMM 3740	*Mangifera indica*	Brazil	KC702921	-	KJ155452	KC992371	KC598098	-	KC702954	-	KC702978	KM404169
C. endomangiferae	CMM 3814*	*Mangifera indica*	Brazil	KC702922	-	KJ155453	KC992372	KC598113	-	KC702955	-	KC702994	KM404170
C. endophytica	MFLUCC 130417, LC1216	*Pennisetum purpureum*	Thailand	KC692467	-	-	KC810017	-	-	KC832853	-	KC633853	-
C. endophytica	MFLUCC 130418, LC0324*	*Pennisetum purpureum*	Thailand	KF306258	-	-	KC810018	-	-	KC832854	-	KC633854	-
C. endophytica	MFLUCC 130419, LC0327	*Pennisetum purpureum*	Thailand	KC692468	-	-	KC810016	-	-	KC832846	-	KC633855	-
C. fragariae	Bra5	*Coffea* sp.	Brazil	-	-	FR718801	-	-	-	-	-	FR717700	FR719885
C. fragariae	Bra8	*Coffea* sp.	Brazil	-	-	FR718802	-	-	-	-	-	FR717701	FR719886
C. fragariae	CBS 142.31,ICMP 17927	*Fragaria × ananassa*	USA	JX009516	-	JQ807844	JX009592	JX009830	-	JX010024	JX010064	JX010286	JX010373
C. fragariae	CMM 4242	*Musa sp.*	Brazil	**KX093971**	**KX094005**	**KX094320**	**KX094068**	**KX094069**	**KX094111**	**KX094173**	**KX094197**	**KX094238**	**KX094278**

C. fragariae	ICMP 17099	*Fragaria × ananassa*	USA	JX009493	-	-	JX009588	JX009778	-	JX009957	-	JX010285	
C. fructicola	1087	*Theobroma cacao*	Panama	**KX093974**	GU994409	GU994438	**KX094066**	**KX094104**	**KX094121**	**KX094174**	**KX094198**	GU994377	**KX094279**
C. fructicola	3589	*Theobroma cacao*	Panama	**KX093975**	GU994411	GU994440	**KX094067**	**KX094105**	**KX094122**	**KX094175**	**KX094199**	GU994376	**KX094280**
C. fructicola	3679	*Theobroma cacao*	Panama	-	GU994410	GU994439	-	-	-	-	-	GU994375	GU994468
C. fructicola	7574	*Theobroma cacao*	Panama	-	GU994413	GU994442	-	-	-	-	-	GU994374	GU994471
C. fructicola	CBS 125397*, ICMP 18646	*Tetragastris panamensis*	Panama	JX009581	GU994412	JQ807839	JX009674	JX009874	-	JX010032	JX010099	JX010173	JX010409
C. fructicola	ColI1126	*Vaccinium macrocarpon*	USA	-	JX145239	JX145315	-	-	-	-	-	JX145136	JX145187
C. fructicola	ColI996	*Rhexia virginica*	USA	-	JX145269	JX145324	-	-	-	-	-	JX145167	JX145219
C. fructicola	ColIP1	*Vaccinium corymbosum*	USA	-	JX145273	JX145316	-	-	-	-	-	JX145171	JX145223
C. fructicola	GM567	*Mangifera indica*	India	JQ894543	-	JQ894576	KC790787	JQ894614	-	JQ894630	-	JQ894676	JQ894600
C. fructicola	ICMP 18581*, CBS 130416	*Coffea arabica*	Thailand	FJ907426	-	JQ807838	FJ917508	JX009866	-	JX010033	JX010095	JX010165	JX010405
C. gloeosporioides	IMI 356878*, ICMP 17821, CBS 112999	*Citrus sinensi*	Italy	JX009531	GU994416	JQ807843	JX009731	JX009818	-	JX010056	JX010085	JX010152	JX010445
C. gloeosporioides f. stylosanthis (syn. *C. fragariae*)	MUCL 42294*, ICMP 17957, CBS 124251	*Stylosanthes viscosa*	Australia	JX009575	-	-	JX009597	JX009821	-	JX009962	JX010063	JX010289	JX010380
C. grevileae	CBS 132879*, CPC 15481	*Grevillea* sp.	Italy	KC296941	-	-	KC296963	KC296987	-	KC297010	KC297033	KC297078	KC297102
C. hebeiense	JZB 330024	*Vitis vinifera*	China	KF377542	-	-	-	-	-	KF377505	-	KF156873	
C. hebeiense	JZB 330028*	*Vitis vinifera*	China	KF377532	-	-	-	KF289008	-	KF377495	-	KF156863	KF288975
C. horii	ICMP 10492*	*Diospyros kaki*	Japan	JX009438	-	JQ807840	JX009604	JX009752	-	GQ329681	JX010137	GQ329690	JX010450
C. hymenocallidis	ICMP 18642*	*Hymenocallis americana*	China	GQ856775	-	JQ807842	JX009709	GQ856730	-	JX010019	JX010100	JX010278	JX010410
C. jasmini-sambac	CLTA B8401	*Jasminum sambac*	Vietnam	HM131510	-		HM131496		-			HM131515	HM153772
C. jasmini-sambac	ICMP 19118*	*Jasminum sambac*	Vietnam	HM131507	-	JQ807841	JX009713	JX009895	-	HM131497	JX010105	HM131511	JX010415
C. melanocaulon	CBS133123	*Vaccinium macrocarpon*	USA	**KX093986**	JX145245	JX145309	**KX094035**	**KX094109**	**KX094133**	**KX094186**	**KX094227**	JX145142	**KX094289**
C. melanocaulon	CBS133251	*Vaccinium macrocarpon*	USA	**KX093987**	JX145247	JX145313	**KX094036**	**KX094110**	**KX094134**	**KX094187**	**KX094228**	JX145144	**KX094290**
C. murrayae	GZAAS 5.09506*	*Murraya* sp.	China	JQ247657	-	-	JQ247596	-	-	JQ247609	JQ247621	JQ247633	JQ247644

C. murrayae	GZAAS 5.09538	*Murraya* sp.	China	JQ247656	-	-	JQ247597	-	-	JQ247608	JQ247620	JQ247632	JQ247645
C. musae	CBS 116870*, ICMP 19119	*Musa* sp.	USA	JX009433	-	KC888926	JX009742	JX009896	-	JX010050	JX010103	JX010146	HQ596280
C. musae	CMM 4421	*Musa* sp.	Brazil	**KX093970**	**KX094025**	**KX094335**	**KX094030**	**KX094090**	**KX094118**	**KX094194**	**KX094237**	**KX094259**	**KX094297**
C. musae	CMM 4422	*Musa* sp.	Brazil	**KX093965**	**KX094011**	**KX094332**	**KX094029**	**KX094075**	**KX094113**	**KX094189**	**KX094232**	**KX094244**	**KX094298**
C. musae	CMM 4423	*Musa* sp.	Brazil	**KX093964**	**KX094010**	**KX094328**	**KX094028**	**KX094074**	**KX094119**	**KX094195**	**KX094231**	**KX094243**	**KX094294**
C. musae	CMM 4445	*Musa* sp.	Brazil	**KX093963**	**KX094008**	**KX094329**	**KX094027**	**KX094072**	**KX094112**	**KX094188**	**KX094230**	**KX094241**	**KX094293**
C. musae	CMM 4447	*Musa* sp.	Brazil	**KX093968**	**KX094017**	**KX094331**	**KX094033**	**KX094082**	**KX094116**	**KX094192**	**KX094235**	**KX094251**	**KX094296**
C. musae	CMM 4450	*Musa* sp.	Brazil	**KX093966**	**KX094012**	**KX094334**	**KX094031**	**KX094076**	**KX094114**	**KX094190**	**KX094233**	**KX094245**	**KX094295**
C. musae	CMM 4452	*Musa* sp.	Brazil	**KX093969**	**KX094019**	**KX094330**	**KX094034**	**KX094084**	**KX094117**	**KX094193**	**KX094236**	**KX094253**	**KX094291**
C. musae	CMM 4458	*Musa* sp.	Brazil	**KX093967**	**KX094016**	**KX094333**	**KX094032**	**KX094080**	**KX094115**	**KX094191**	**KX094234**	**KX094249**	**KX094292**
C. musae	GB07	*Musa* sp.	India	KC790615	-	KC790667	KF451948	KF451983	-	KC790729	-	KC790968	-
C. musae	GB15	*Musa* sp.	India	KC790616	-	KC790668			-	KC790730	-	KC790969	-
C. musae	GBM03	*Musa* sp.	India	KC790618	-	KC790670	KF451950	KF451985	-	KC790732	-	KC790971	-
C. musae	IMI 52264, ICMP 17817	*Musa sapientum*	Kenya	JX009432	-	-	JX009689	JX009815	-	JX010015	-	JX010142	JX010395
C. nupharicola	CBS 469.96, ICMP 17938	*Nuphar lutea subsp. polysepala*	USA	JX009486	-	-	JX009661	JX009834	-	JX009936	JX010087	JX010189	JX010397
C. nupharicola	CBS 470.96*, ICMP 18187	*Nuphar lutea subsp. polysepala*	USA	JX009486	JX145275	JX145319	JX009661	JX009834	-	JX009936	JX010088	JX010189	JX010397
C. nupharicola	CBS 472.96, ICMP 17940	*Nymphaea odorata*	USA	JX009582	JX145276	JX145320	JX009662	JX009836	-	JX010031	JX010089	JX010188	JX010399
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX009447	-	KC888928	JX009691	JX009899	-	JX009934	JX010104	JX010276	JX010414
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	JX009562	-	KC888925	JX009696	JX009863	-	JX009916	JX010093	JX010242	JX010403
C. siamense	ICMP 18578*, CBS 130417	*Coffea arabica*	Thailand	FJ907423	-	JQ899289	FJ917505	JX009865	-	JX009924	JX010094	JX010171	JX010404
C. siamense	Thai4	*Coffea arabica*	Thailand	-	-	HE655654	-	-	-	-	-	HE655514	HE655600
C. theobromicola	CBS 124945*, ICMP 18649	*Theobroma cacao*	Panama	JX009444	GU994419	KC790726	JX009591	JX009869	-	JX010006	JX010139	JX010294	JX010447
C. theobromicola	GJS B1160843	*Theobroma cacao*	Panama	-	GU994418	GU994447	-	-	-	-	-	GU994356	GU994476
C. theobromicola	ICMP 17895	*Annona diversifolia*	Mexico	JX009568	-	-	JX009600	JX009828	-	JX010057	JX010066	JX010284	JX010382

C. tropicale	CBS 124949*, ICMP 18653	*Theobroma cacao*	Panama	JX009489	GU994396	GU994425	JX009719	JX009870	-	JX010007	JX010097	JX010264	GU994454
C. tropicale	CMM 3767	*Mangifera indica*	Brazil	KC702928	-	KJ155464	KC992378	KC992388	-	KC702960	-	KC702985	KC992345
C. tropicale	CMM 3780	*Mangifera indica*	Brazil	KC702926	-	KJ155467	KC992374	KC992386	-	KC702961	-	KC702972	KC992343
C. tropicale	CMM 3783	*Mangifera indica*	Brazil	KC702927	-	KJ155465	KC992375	KC992387	-	KC702958	-	KC702975	KC992344
C. tropicale	CMM 3787	*Mangifera indica*	Brazil	KC702923	-	KJ155466	KC992376	KC992383	-	KC702956	-	KC702962	KC992340
C. tropicale	CMM 4073	*Mangifera indica*	Brazil	KC533730	-	-	KC517217	-	-	KC517185	KC430870	KC329788	KC517262
C. tropicale	CMM 4074	*Mangifera indica*	Brazil	KC533728	-	-	KC517215	-	-	KC517183	KC430867	KC329786	KC517260
C. tropicale	CMM 4243	*Musa sp.*	Brazil	KU213596	KU213598	KU213597	KU213599	KU213600	**KX094120**	KU213601	KU213602	KU213603	KU213604
C. tropicale	Coll B80918	*Terpsichore taxifolia*	Puerto Rico	-	JX145264	JX145307	-	-	-	-	-	JX145162	JX145214
C. viniferum	GZAAS 5.08601*	*Vitis vinifera*	China	JN412795	-	-	JQ309639	-	-	JN412798	-	JN412804	JN412813
C. viniferum	GZAAS 5.08608	*Vitis vinifera*	China	JN412793	-	-	JN412782	-	-	JN412800	-	JN412802	JN412811
Colletotrichum sp. (*C. siamense s. l.*)	7767	*Theobroma cacao*	Panama	-	GU994403	GU994432	-	-	-	-	-	GU994337	GU994461
Colletotrichum sp. (*C. siamense s. l.*)	CMM 4244	*Musa sp.*	Brazil	**KX093985**	**KX094014**	**KX094315**	**KX094055**	**KX094078**	**KX094135**	**KX094172**	**KX094226**	**KX094247**	**KX094299**
Colletotrichum sp. (*C. siamense s. l.*)	CMM 4247	*Musa sp.*	Brazil	**KX093973**	**KX094009**	**KX094301**	**KX094038**	**KX094073**	**KX094141**	**KX094155**	**KX094196**	**KX094242**	**KX094261**
Colletotrichum sp. (*C. siamense s. l.*)	CMM 4248	*Musa sp.*	Brazil	**KX093972**	**KX093992**	**KX094314**	**KX094037**	**KX094081**	**KX094136**	**KX094154**	**KX094229**	**KX094250**	**KX094300**
Colletotrichum sp. (*C. siamense s. l.*)	GJS 0852	*Theobroma cacao*	Panama	-	GU994404	GU994433	-	-	-	-	-	GU994342	GU994462
Colletotrichum sp. (*C. siamense s. l.*)	GN01	*Azadiractha indica*	India	KC790621	-	KC790673	KF451952	KF451987	-	KC790735		KC790974	KC790868

1. CBS = Centraalbureau voor Schimmelcultures (Netherlands), ICMP = International Collection of Microorganisms from Plants, MFLUCC = Mae Fah Luang University, IMI = CABI Genetic Resource Collection (UK), MAFF = Ministry of Agriculture, Forestry and Fisheries (Japan), GZAAS = Guizhou Academy of Agricultural Sciences herbarium (China), CMM = Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco (Brazil), GJS = culture collection G.J. Samuels, United States Department of Agriculture (USA), GUFCC = Goa University Fungal Culture Collection (India), MUCL = Belgian Co-ordinated Collections of Micro-organism, (agro)industrial fungi and yeasts (Belgian), URM = Culture Collection Micoteca URM, Department of Mycology, Universidade Federal de Pernambuco (Brazil). "*" indicates ex-holotype or ex-epitype cultures. 2. Sequences generated in the present study are emphasized in bold. "-"indicates missing data.

Table 2. Summary statistics for each MSA after manual adjustment or after alignment filtering and the topological distance between the tree inferred from the manually adjusted alignment and all other alignment filtering approaches.

Individual datasets	Taxa	Length	V	PI	MS	R-F
ACT E	98	294	62	38	-	-
ACT G		257	52	36	9	2
ACT M		294	59	35	5	1.5
ACT T		284	55	32	34	3
APN2 E	60	829	139	101	-	-
APN2 G		806	135	100	0	0
APN2 M		829	139	100	2	1
APN2 T		824	136	98	0	0
APN2/MAT-IGS E	89	968	448	285	-	-
APN2/MAT-IGS G		741	370	265	4	0.5
APN2/MAT-IGS M		968	416	279	4	2
APN2/MAT-IGS T		955	445	281	23	1.5
CAL E	93	765	142	96	-	-
CAL G		662	127	87	49	2.5
CAL M		765	142	96	9	2.5
CAL T		760	139	94	5	0.5
CHS-1 E	84	299	30	15	-	-
CHS-1 G		268	30	14	0	0
CHS-1 M		-	-	-	-	-
CHS-1 T		-	-	-	-	-
GAP2-IGS E	43	899	146	60	-	-
GAP2-IGS G		878	145	60	0	0
GAP2-IGS M		899	144	59	21	0.5
GAP2-IGS T		855	130	53	0	0
GAPDH E	95	1035	129	91	-	-
GAPDH G		231	98	74	14	1.5
GAPDH M		1035	129	91	61	15.5
GAPDH T		1014	121	86	63	16
GS E	74	945	210	135	-	-
GS G		785	177	117	26	3
GS M		945	205	135	1	1
GS T		928	202	131	0	0
ITS E	108	600	21	12	-	-
ITS G		538	19	12	2	1
ITS M		600	21	12	3	1
ITS T		581	20	11	4	1
TUB2 E	103	1507	229	163	-	-
TUB2 G		875	154	108	38	8.5
TUB2 M		1507	217	160	23	5.5
TUB2 T		1450	195	146	39	5.5

Table 2. (Ctd).

Combined dataset	Taxa	Length	V	PI	MS	R-F
10 loci E	108	8141	1556	996	-	-
10 loci G		6041	1307	873	112	12.5
10 loci M		8141	1502	982	32	8
10 loci T		7950	1473	947	114	18.5

*E - manualy edited; G - Gblocks with relaxed selection; M - residues masked by GUIGANCE2; T - columns removed by GUIDANCE2. Length. Number of characters (bp) in the alignment. V - Number of variable characters (bp). PI. Number of parsimony informative characters (bp). MS - Matching Split distance. R-F - Robinson-Foulds distance. The ML tree inferred from the manualy edited alignment was used as the reference tree.

Table 3. Summary statistics for the phylogenetic informativeness profiles of 13 amplicons in the *C. gloeosporioides* species complex.

Loci	Length	V	PI	%PI	Maximum NIV (10^{-3})	Maximum PSIV	Maximum values reached at*
ACT	259	23	2	0.77	15.24	59.07	0.38
APN2	826	108	37	4.48	93.31	112.97	0.19
APN2/MAT-IGS	790	221	76	9.62	163.57	207.58	0.29
CAL	665	68	18	2.71	48.50	73.27	0.25
CHS-1	268	15	2	0.75	9.81	36.62	0.14
GAP2-IGS	899	137	28	3.11	97.04	110.28	0.26
GAPDH-972 (GAP-95+GAP-1174)	972	71	14	1.44	52.36	54.14	0.29
GAPDH-208 (GDF+GDR)	208	46	6	2.88	30.30	149.24	0.38
GS	787	98	24	3.05	69.44	88.34	0.27
ITS	556	9	5	0.90	24.81	44.77	0.06
TUB2-1460 (T1+T22)	1460	140	21	1.44	91.26	62.98	0.47
TUB2-702 (T1+Bt2b)	702	73	8	1.14	49.51	71.65	0.45
TUB2-444 (Bt2a+Bt2b)	444	36	4	0.90	23.23	52.80	0.50

Length. Number of characters (bp) in the alignment. Variable. Number of variable characters (bp). PI. Number of parsimony informative characters (bp). %PI. Percentage of parsimony informative characters in the alignment. NIV. Net informativeness values. PSIV. Per site informativeness values. * = Values are expressed in arbitrary units.

Table 4. Pairwise comparison of the mean conidial length, width and length/width ratio produced on CMA, PDA and SNA.

Colletotrichum species pairwises	CMA			PDA			SNA		
	Length	Width	Length/width ratio	Length	Width	Length/width ratio	Length	Width	Length/width ratio
C. dianesei × *C. aureum*									
C. fragariae × *C. aureum*									
C. fragariae × *C. dianesei*									
C. musae × *C. aureum*									
C. musae × *C. dianesei*									
C. musae × *C. fragariae*									
C. tropicale × *C. aureum*									
C. tropicale × *C. dianesei*									
C. tropicale × *C. fragariae*									
C. tropicale × *C. musae*									
C. tropicale × *Colletotrichum* sp. 2									
Colletotrichum sp. 2 × *C. aureum*									
Colletotrichum sp. 2 × *C. dianesei*									
Colletotrichum sp. 2 × *C. fragariae*									
Colletotrichum sp. 2 × *C. musae*									

Means were compared by the Tukey's HSD test at 95% of confidence level. Light grey color indicates significant difference ($p \leq 0.05$) and dark grey color indicates non-significant difference ($p > 0.05$) between means.

Capítulo III

Thiophanate-methyl Resistance and Fitness Components of *Colletotrichum musae* Isolates From Banana in Brazil

Thiophanate-methyl Resistance and Fitness Components of *Colletotrichum musae* Isolates From Banana in Brazil

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Resumo

A antracnose, causada por *Colletotrichum musae*, é a doença pós-colheita mais importante da banana e está amplamente distribuída em todas as regiões de produção de banana no Brasil. No entanto, não há dados disponíveis sobre a sensibilidade de *C. musae* ao tiofanato-metílico, o fungicida mais comumente utilizado em bananais no Brasil. Com base no teste in vitro de sensibilidade ao tiofanato-metílico, existem vários isolados classificados como moderadamente-resistentes, cuja concentração do fungicida em que o desenvolvimento dos fungos é inibida em 50% variou de 10,43 a 48,73 ug de ingrediente ativo por mililitro. Isolados moderadamente-resistentes apresentam uma substituição de TAC para TTC no códon 200 do gene β-tubulina. A aplicação do tiofanato-metílico na sua dose comercial apresentou baixa eficácia no controle dos isolados moderadamente-resistentes em bananas. Não foram observadas diferenças estatísticas entre os isolados sensíveis e moderadamente resistentes para os componentes da adaptabilidade avaliados (P> 0,05), indicando a ausência de custo adaptativo para isolados moderadamente resistentes.

Abstract

Anthracnose, caused by *Colletotrichum musae*, is the most important post-harvest disease of banana and is widely distributed among the banana production regions in Brazil. However, there are no data available on the sensitivity of *C. musae* to thiophanate-methyl, the most common fungicide used in banana orchards in Brazil. Based on the in vitro thiophanate-methyl sensitivity test, there are several isolates moderately resistant to the concentration of the fungicide at which fungal development is inhibited by 50%, ranging from 10.43 to 48.73 μg/ml. Moderately resistant isolates carried a substitution of TAC for TTC at codon 200 in the β-tubulin gene. Thiophanate-methyl application at the label rate showed low efficacy in controlling the moderately resistant isolates on banana fruit. No statistical differences were observed between the sensitive and moderately resistant isolates for the fitness components evaluated ($P>0.05$), indicating the absence of fitness cost for moderately resistant isolates.

Brazil is the fifth-largest producer of banana (*Musa* spp. L.) worldwide, just surpassed by India, China, the Philippines and Ecuador. However, fruit quality is impacted by diseases caused by post-harvest pathogens, which limits the exportability of Brazilian fruit, preventing long-distance transport and entry to its final destination (Prusky and Plumbley 1992; Peres, 1998). Banana anthracnose caused by *Colletotrichum musae* is the most important postharvest disease of the crop, and is present in all production areas worldwide. The disease can occur at any time during the growing season and appears mainly on banana fruit (Simmonds and Mitchell 1940). The pathogen starts as a quiescent infection on green fruit (Lim et al. 2002), but the typical anthracnose symptoms generally can only be seen in overripe fruit or fruit ripened at high temperatures (Meredith 1960).

Banana anthracnose management consists of practices to prevent infection and delay symptom development. The control must start in the field with good cultural practices such as the frequent defoliation of banana and removal of crop debris, which is the main way to combat the inoculum at its source (Zambolim et al. 2002). During harvest it is necessary to prevent injuries on the fruit surface (Codeiro 1997). After harvest, fungicides, especially benzimidazoles, are commonly used for control of anthracnose (Eckert and Ogawa 1985).

In Brazil, thiabendazole is the only fungicide of the methyl benzimidazoles carbamate (MBC) group registered for banana postharvest treatment (MAPA 2014). However, thiophanate-methyl, another active ingredient belonging to MBC group, is commonly applied to banana orchards in Brazil and is registered for control of yellow Sigatoka leaf spot caused by *Mycosphaerella musicola* (MAPA 2014). Although thiophanate-methyl is not used in the field for control of anthracnose, *C. musae* populations are exposed to the fungicide. Thus, there is an expectation that populations of *C. musae* are resistant to thiophanate-methyl in Brazilian banana orchards.

Among several factors that can lead to unfavorable results when plant diseases are managed with fungicides, the most important is the reduction in sensitivity of the target fungus. This reduction in the sensitivity has become commonplace after the introduction of fungicides with a specific mode of action (Ma and Michailides 2005; Brent and Hollomon 2007a, b; Avenot and Michailides 2010; Kuck et al. 2012). MBCs act by inhibiting nuclear division by binding to β-tubulin subunits (Davidse 1986; Koller 1999). Resistance to MBC group fungicides has been detected in many fungal species and is commonly associated with point mutations in the β-tubulin gene (TUB2), which result in changes in amino acid sequences at the binding site (Chung et al. 2010; Ma and Michailides 2005). Although changes at codon 6, 50, 167, 198, 200, and 240 in the TUB2 gene could cause MBC resistance in field isolates of pathogenic fungi (Ma and Michailides 2005), mutations at positions 198 and 200 are most commonly detected in *Colletotrichum* isolates (Peres et al. 2004, Maymon et al. 2006; Wong et al. 2008; Chung et al. 2010; Kongtragoul et al. 2011, Hu et al. 2015). The mutation in codon 198 is commonly found in isolates with high levels of resistance, while the mutation at position 200 is correlated with intermediate levels (Chung et al. 2006, 2010).

Fungicide resistance is a stable, heritable adjustment by a fungus to a fungicide, resulting in reduced sensitivity of the fungus to specific fungicide dosages that previously provided effective control. However, resistance may have a fitness cost (Ma and Michailides 2005). Fitness can be defined as the ability of a fungal isolate to develop, reproduce, survive and cause disease, compared to other isolates under the same conditions. The mycelial growth, reproductive potential and virulence are some examples of fitness components evaluated in populations of plant pathogens (Antonovics and Alexander 1989; Peever and Milgroom 1994; Zhan and Mcdonald 2013; Milgroom 2015). When resistant individual have

a significant fitness cost in comparison with the sensitive ones, they should decline sooner in the absence of fungicide selection pressure (Mikaberidze and McDonald 2015).

Since fungicides are used for the control of banana diseases in Brazil, it is necessary to investigate the sensitivity of *C. musae* to the main fungicides employed in the field in order to adopt the best management measures. Thus, the objectives of the present study were to i) characterize the sensitivity of C. musae isolates from Brazilian banana orchards to thiophanate-methyl, ii) evaluate the efficacy of fungicide in controlling isolates with different levels of sensitivity in banana fruit, iii) investigate molecular mechanisms of resistance, and (iv) determine the relationship between the levels of sensitivity to this fungicide and fitness parameters.

Materials and Methods

Sampling and Isolation. In 2012 and 2013, samples of the banana cultivars 'Ouro', 'Nanica', 'Nanicão', 'Caturra', 'Caru-roxa', 'D'água', 'Prata', 'Pacovan', 'Maçã' and 'Terra' were collected from orchards located in the following Brazilian States: Alagoas, Bahia, Distrito Federal, Espírito Santo, Goiás, Minas Gerais, Para, Paraná, Pernambuco, Santa Catarina and São Paulo. One unripe fruit was collected from a minimum of 10 plants in each orchard with each sampling point separated by at least 20 meters. Fruit were returned to the lab, washed in running water and kept in a humid chamber for two days. The fruit were then removed from the humid chamber and maintained in the lab and monitored for the appearance of lesions. Conidia from developing acervuli were transferred to Petri dishes containing potato-dextrose-agar (PDA – Himedia, India), and isolates similar in morphology to the description of *C. musae* (Su et al. 2011, Weir et al. 2012) were recorded. Single conidium

isolates were preserved on PDA slants at 4 °C and at room temperature in cryogenic tubes containing autoclaved distilled water.

The isolates morphologically identified as *C. musae* were tested with the *C. musae* species-specific primers Musae_tub_33F and Musae_tub_387 (Vieira et al., *submitted*) to confirm their identity. Genomic DNA was extracted following the CTAB (cetyl trimethyl ammonium bromide) protocol (Doyle and Doyle, 1990) with slight modifications. PCR reactions were performed using the following cycling parameters: initial denaturing for 3 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. The amplification products were examined by agarose gel electrophoresis and the *C. musae* isolates were identified by the presence of a single amplicon of approximately 350 bp.

Thiophanate-methyl sensitivity in vitro assay., *Colletotrichum musae* isolates were screened by a mycelial growth assay using a commercial formulation of thiophanate-methyl (Cercobin 700 WP, 700 g kg^{-1} active ingredient, Iharabras, São Paulo, SP, Brazil) to quantify levels of sensitivity to thiophanate-methyl in vitro. Five-millimeter-diameter plugs were taken from the expanding margin of colonies grown for seven days on PDA, and transferred to the center of PDA plates amended with Cercobin 700 WP at the following active ingredient concentrations: 0 (control), 0.1, 0.2, 0.3, 0.3, 0.5, 1, 2, 3, 4, 10, 50, 100 and 500 μg/ml. Each isolate was plated to three replicate plates at each fungicide concentration and incubated at 25 °C in the dark. Two orthogonal measurements were taken from the colonies after four days of incubation. The percentage of mycelial growth inhibition compared to the control was calculated for all fungicide concentrations, and the concentration of the fungicide at which fungal development was inhibited 50% (EC_{50}) was determined for each isolate. The isolates were classified into three fungicide reaction phenotypes according to EC_{50} range: sensitive for EC_{50} below 10 μg/ml; moderately resistant for EC_{50} between 10 and 100 μg/ml; and highly

143 resistant for EC_{50} above 100 μg/ml. Representative isolates from each fungicide reaction
144 phenotype were selected for the subsequent assays.
145
146 **Thiophanate-methyl sensitivity in vivo assay.** The level of sensitivity of *C. musae*
147 isolates to thiophanate-methyl *in vivo* was assessed by quantifying disease severity on banana
148 fruit treated with the commercial formulation of thiophanate-methyl at the label rate. Banana
149 fruit of the cultivar 'Pacovan' at stage four of maturation (Ministério da Integração Nacional
150 2000) untreated with fungicides were washed in running water, surface disinfected in 1 %
151 NaOCl for 3 min, then rinsed in sterile distilled water. The fruit were wounded at two points
152 by pricking the surface with a pin to a depth of 3 mm. The commercial formulation of
153 thiophanate-methyl was solubilized in sterile distilled water at the label rate (500 μg of active
154 ingredient per milliliter). The wounded fruit were completely immersed in the fungicide
155 solution for 10 s and then kept at room temperature until completely dried. Untreated fruit
156 were immersed in distilled water. Treated and untreated fruit were inoculated with 40 μL of a
157 spore suspension (1×10^6 spores/ml) on the wounds, and kept in a humid chamber at 25°C.
158 The humid chamber was removed 24 h after inoculation and the fruit were maintained at the
159 same temperature. Each replicate was represented two lesions on a single fruit with three
160 replicates per isolate and disease severity was assessed by calculating the mean diameter of
161 the lesions. Differences in disease severity on treated and untreated fruit were determined for
162 each fungicide reaction phenotype by a one-way analyses of variance (ANOVA) and means
163 of each treatment were compared by the Student's t-test (P=0.05) using the software Statistix
164 v.10.
165
166 **Analysis of thiophanate-methyl resistance using partial sequence of β-tubulin**
167 **gene.** To investigate the relationship between genetic variability and different levels of

sensitivity to thiophanate-methyl, partial sequences of the β-tubulin gene (TUB2) were analyzed. The PCR and sequence primers T1 and T22 (O'Donnell and Cigelnik 1997) and the internal sequencing primers Coll_Bt_F1int and Coll_Bt_R1int (Rojas et al. 2010) were used to amplify and sequence approximately 1330 bp. This set of primers was chosen in order to amplify TUB2 sequences that contain several mutation points associated with different levels of benzimidazole resistance in *Colletotrichum* (Chung et al. 2006, 2010; Hu et al. 2015; Kongtragoul et al. 2011) and other plant pathogens (Albertini et al. 1999; Baraldi et al. 2003; Gafur et al. 1998; Koenraadt et al. 1992; Ma et al. 2003; Orbach et al. 1986; Yan and Dickman 1996; Yarden and Katan 1993). The PCR reactions were performed using the following parameters: initial denaturing for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min and 30 s, followed by a final extension at 72 °C for 10 min. PCR products were purified and sequenced on an ABI 3730*xl* (Applied Biosystems) at Beckman Coulter Genomics (Danvers, Massachusetts, USA).

Sequence reads were assembled into contigs and edited in Geneious 8.1. A multiple sequence alignment (MSA) with the sequences generated in our study was built with the online implementation of MAFFT version 7 (Katoh and Toh 2013) using the G-INS-i iterative refinement method. The TUB2 sequence of a wild-type *Colletotrichum* isolate (GenBank accession number U14138), which contains the complete CDS of TUB2, was included in the alignment and used as reference for the codon position assignment and detection of point mutations. Polymorphic sites in the alignment were detected using MEGA 6.06 (Tamura et al. 2013).

Analysis of fitness components. The following fitness components were determined for the *C. musae* isolates with different levels of resistance to thiophanate-methyl: mycelial growth rate, spore production in vitro, spore germination, osmotic sensitivity and virulence.

For all assays, each isolate was first transferred to PDA plates (five plates per isolate) and incubated for seven days at 25 °C with continuous fluorescent light to enhance sporulation. All assays were conduced with three replicates per isolate.

Five-millimeter-diameter plugs were taken from the edge of seven days old colonies, and transferred to the center of the PDA plates to measure the mycelial growth rate. Each isolate was plated to three replicate plates and incubated as described above. Four radial measurements were taken from the edge of the plug to the margin of the colony after four days of incubation, resulting in four radial measurements per replicate to calculate the mycelial growth rate (mm/day).

Colonies with active sporulation were rinsed with 20 ml of distilled water and the conidial suspension was filtered through double-layered cheesecloth to quantify spore production. The spore concentration in the suspension was measured with the aid of a hemacytometer and expressed as number of spores per milliliter of suspension. The concentration of the solutions were then adjusted to 1×10^6 spores/ml to be used in the spore germination and virulence assays.

Fifty microliters of spore suspension were transferred to four equidistant points on 2% water agar Petri plates to calculate the spore germination rates. After 12 h of incubation at 25 °C in the dark, 100 spores under each coverslip were examined with the aid of a compound microscope (Olympus BX41, Olympus Co., Tokyo, Japan) to determine the germination percentage. A spore was considered germinated if the germ tube was at least half the length of the spore.

Five-millimeters-diameter plugs were taken from the expanding margins of colonies grown for seven days on PDA, and transferred to the center of the PDA plates amended with 0 (control) 1, 2, 4, 6 or 8 % (wt/vol) NaCl to determine osmotic sensitivity. Each isolate was plated to three replicate plates at each NaCl concentration and incubated at 25 °C in the dark.

Two orthogonal measurements were taken from the colonies after four days of incubation. The percentage of mycelial growth inhibition related to the control was calculated for all NaCl concentrations, and the concentration of NaCl that inhibits fungal development to 50% ($EC_{50}N$) was determined for each individual isolate.

The values of disease severity on untreated fruit for each isolate were used from the thiophanate-methyl sensitivity in vivo assay for the virulence analysis. Untreated fruit inoculated with 40 µl of distilled water were used as the negative control.

Analyses of variance (ANOVA) were performed individually for each fitness component and differences among the different fungicide reaction phenotypes were determined. The statistical analyses were performed with Statistix v.10.

Results

Sampling and isolation. A total of 139 *Colletotrichum* isolates similar in morphology to *C. musae* were obtained from the different sampled areas. All isolates produced a single amplicon of approximately 350 bp with the *C. musae* species-specific primers, confirming the assignment of these isolates to *C. musae*.

Thiophanate-methyl sensitivity in vitro assay. Differences were observed in the sensitivity response to thiophanate-methyl among the *C. musae* isolates. The EC_{50} values ranged from 0.003 to 48.73 and the isolates were grouped into two fungicide reaction phenotypes. One hundred thirty three isolates (96%) had EC_{50} values ranging from 0.003 to 4.84 µg/ml and were classified as sensitive to thiophanate-methyl, while the remaining six isolates (4%) had EC50 values ranging from 10.43 to 48.73 µg/ml and were classified as

242 moderately resistant. Four isolates of each fungicide reaction phenotype were selected for
243 subsequent assays.
244
245 **Thiophanate-methyl sensitivity in vivo assay.** The efficacy of thiophanate-methyl in
246 controlling *C. musae* was dependent on the sensitivity of the isolates (Table 1). Banana fruit
247 untreated with fungicide and inoculated with sensitive isolates develop typical anthracnose
248 symptoms, while no symptoms were observed on fruit treated with the fungicide. When the
249 fruit were inoculated with moderately resistant isolates, disease severity significantly
250 decreased in the treated fruit compared with the untreated fruit ($P \leq 0.05$). The commercial
251 formulation of thiophanate-methyl at the label rate efficiently controlled banana anthracnose
252 when the fruit was inoculated with the sensitive isolates, while disease severity was reduced
253 but not eliminated when fruit was inoculated with the resistant isolates.
254
255 **Detection of mutations associated with thiophanate-methyl resistance using**
256 **partial sequence of β-tubulin gene.** *Colletotrichum musae* isolates with different levels of
257 thiophanate-methyl resistance carried distinct mutations in the TUB2 region (Table 2).
258 Sensitive isolates were identical in the coding region to the wild-type *Colletotrichum*
259 sequence. In contrast, a transversion from thymine (T) to adenine (A) at position 978 was
260 present in all moderately resistant isolates. This point mutation at codon 200 in the second
261 position results in a substitution of phenylalanine (TTC) (in the wild-type and sensitive
262 isolates) for a tyrosine (TAC) in the moderately resistant isolates and is therefore directly
263 correlated with the moderately resistant phenotype in the *C. musae* isolates.
264

265 **Analysis of fitness components.** No statistical difference was observed between the
266 thiophanate-methyl sensitive and resistant isolates for mycelial growth rate, spore production,
267 spore germination, osmotic sensitivity and virulence on banana fruit (Table 3).
268
269 **Discussion**
270
271 This is the first report on the sensitivity of *C. musae* isolates from banana orchards to
272 thiophanate-methyl and its influence on fitness components in sensitive and resistant isolates.
273 Previous studies have reported resistance to other MBC fungicides in *C. musae* populations
274 from other countries (Grifee 1973; Quimio 1976, Slabaugh and Grove 1982; Hostachy et al.
275 1990; Johhanson and Blasquex 1992; de Lapeyre de Bellaire and Dubois 1997).
276 The in vitro sensitivity test revealed that most of the *C. musae* isolates are thiophanate-
277 methyl sensitive. In most of the sampled area a regular schedule of fungicide application has
278 not been adopted, due to the low cost-benefit. All moderately resistant isolates originated
279 from the same orchard in Registro, São Paulo. Among the 13 isolates from this orchard, six
280 were classified as moderately resistant to thiophanate-methyl. Distinct from the other sampled
281 areas, regular applications of fungicide are used for disease control during banana production
282 in Registro. This is largely due to the cultivation of 'Prata' in the sampled orchard, a cultivar
283 that is highly susceptible to yellow Sigatoka leaf spot and therefore requires an intensive use
284 of fungicides to control this disease. Thiophanate-methyl is one of the fungicides registered
285 for the control of Sigatoka in Brazil (MAPA 2016) and has been adopted in the sampled
286 orchard. Although thiophanate-methyl is not registered for anthracnose control, the *C. musae*
287 population is exposed to the action of the fungicide in the field and has likely led to the
288 emergence and success of moderately resistant isolates.

289 The rate of change caused by resistance is correlated with the epidemiology of the
290 pathogen and the frequency or duration of the selection pressure applied (Ma and Michailides
291 2005, Brent and Hollomon 2007a, b; Mikaberidze and McDonald 2015; Milgroom 2015).
292 Fungicide resistant alleles are often rare or nonexistent in populations of fungi never subjected
293 to fungicide applications. However, after application of fungicides, these alleles can emerge
294 and increase their proportion in the population (Brent and Hollomon 2007a, b). Factors such
295 as increasing efficacy of the fungicide, increasing fungicide application, and a greater
296 difference in sensitivity between resistant and sensitive individuals in the field can lead to
297 greater selection pressure (Bergamin Filho and Amorim 2001). Therefore, the *C. musae*
298 population from Registro, where the thiophanate-methyl moderately resistant isolates were
299 found, may have been subjected to selection pressure over time by the frequent use of this
300 fungicide to control of *M. musicola*.

301 The test of fungicide resistance in vivo explored the field relevance of the presence of
302 *C. musae* isolates with different fungicide resistance levels by the efficacy of thiophanate-
303 methyl in controlling them at the commercial dosage. Our results revealed the low efficacy of
304 thiophanate-methyl in controlling moderately resistant isolates of *C. musae* in the field. It has
305 significant practical implications for postharvest disease control, since the moderately
306 resistant isolates were collected in one of the main Brazilian areas of banana production for
307 export.

308 Our analysis of the TUB2 region showed that the single-base-pair mutation converting
309 codon 200 from phenylalanine to tyrosine was observed in the moderately resistant but not in
310 the sensitive isolates of *C. musae* from banana. The same association has been reported in *C.*
311 *gloeosporioides* (Chung et al. 2006, 2010) and in other plant pathogenic fungi (Koenraadt et
312 al. 1992, Yarden and Katan 1993; Albertini et al. 1999). Although different point mutations
313 have been correlated with levels of benzimidazole resistance in fungal isolates (Albertini et al.

1999; Baraldi et al. 2003; Gafur et al. 1998; Koenraadt et al. 1992; Ma et al. 2003; Orbach et al. 1986; Yan and Dickman 1996; Yarden and Katan 1993), no other mutations were observed in TUB2 sequences among the moderately resistant *C. musae* isolates.

Information about fitness components of resistant and sensitive fungi to fungicides is useful to determine disease management strategies and prevent the development of resistance (Antonovics and Alexander 1989; Ma and Michailides 2005). Fungicide resistance is a genetically inherited trait that may impose a fitness penalty, which can negatively affect fungal growth, reproduction, or pathogenicity (Brent and Hollomon, 2007b). Fungicide resistance can be considered a fitness conditioner, since resistant isolates overcome the selective barrier exercised by the use of fungicide, complete the life cycle and surpass the sensitive isolates by leaving more descendants. However, if this resistance has a fitness cost, resistant individuals will have less competitive ability in the absence of the selective barrier, which means that the proportion of sensitive individuals in the population will naturally increase if the fungicide applications in the field are interrupted (Ma and Michailides 2005). This study shows that isolates of *C. musae* moderately resistant to thiophanate-methyl are as competitive as sensitive isolates and equally capable of surviving and persisting in banana orchards. Therefore, moderately resistant isolates of *C. musae* may spread in banana orchards despite the selection pressure imposed by multiple applications of thiophanate-methyl.

Although fungicide resistance management can attenuate the emergence of non-sensitive isolates in field populations (Brent and Hollomon, 2007a), there are no studies on the most appropriate strategies for the management of fungicide resistance in banana orchards. Since sensitive and moderately resistant isolates have the same adaptive potential, the first management strategy to be adopted is the use of other fungicides with action mechanisms distinct from benzimidazoles. Other strategies, such as the application of thiophanate-methyl in mixture with one or more fungicides with different modes of action,

339 application only when strictly necessary and following the dose recommended by
340 manufacturers, and the use of other disease management practices, including removal of crop
341 residues from the field, prevention of injuries on the fruit surface during harvest, and fruit
342 treatment in the postharvest could be adopted to reduce the emergence of fungicide resistance
343 in populations of *C. musae*.
344
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346
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493

494 **Table 1**. Disease severity (lesion diameter) on detached banana fruit treated with formulated
495 thiophanate-methyl at the label rate and inoculated with sensitive and moderately resistant
496 isolates of *Colletotrichum musae*.
497

Reaction Phenotype	Lesion diameter (mm)	
	Without fungicide	With fungicide
Sensitive	16.53 (3.07)	0
Moderately resistant	18.73 (2.83)a	15.81(3.41)b

498
499 a Each reaction phenotype is represented by four isolates
500 b Values on each line (isolate class) followed by the same letter are not significantly different
501 according to Student's t-test (P=0.05). Values (±) in parentheses represent standard errors
502

503 **Table 2.** Partial nucleotide and amino acid sequences of the β-tubulin gene (TUB2) from thiophanate-methyl sensitive and moderately resistant
504 isolates of *Colletotrichum musae* from Brazil.

Isolates	Partial nucleotide and amino acid sequences[a]
Sensitive	
C. musae BA2	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TTC** TGC ATT GAC AAC GAG GCT
C. musae GM20	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TTC** TGC ATT GAC AAC GAG GCT
C. musae MM5	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TTC** TGC ATT GAC AAC GAG GCT
C. musae PN9	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TTC** TGC ATT GAC AAC GAG GCT
C. gloeosporioides f. sp. *aeschynomenes* 3.1.3[b]	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TTC** TGC ATT GAC AAC GAG GCT
Amino acid	Q L V E N S D E T **Y** C I D N E A
Moderately resistant	
C. musae RP3	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TAC** TGC ATT GAC AAC GAG GCT
C. musae RP4	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TAC** TGC ATT GAC AAC GAG GCT
C. musae RP5	CAG CTG GTC GAG AAC TCC GAC GAG ACC **TAC** TGC ATT GAC AAC GAG GCT

C. musae RP6	CAG	CTG	GTC	GAG	AAC	TCC	GAC	GAG	ACC	**TAC**	TGC	ATT	GAC	AAC	GAG	GCT
Amino acid	Q	L	V	E	N	S	D	E	T	**Y**	C	I	D	N	E	A
Codon position	191	192	193	194	195	196	197	198	199	**200**	201	202	203	204	205	206

505 a – Codons with nucleotide substitutions and amino acid changes are highlighted in bold face.

506 b – Partial sequence of the benomyl-sensitive isolate used as reference.

507

Table 3. Comparison of mycelial growth rate (MGR), spore production (SP), spore germination (SG), osmotic sensitivity ($EC_{50}N$) and virulence (lesion diameter) on banana fruit (VIR) between thiophanate-methyl sensitive and moderately resistant isolates of *Colletotrichum musae*.

Reaction phenotype[a]	MGR (mm/day)[b]	SP ($\times 10^6$ conidia/ml)[b]	SG (%)[b]	$EC_{50}N$ (%NaCl)[b]	VIR (mm)[b]
Sensitive	19.31 (0.52)	4.62 (1.25)	63.42 (14.5)	3.89 (0.17)	16.53 (3.07)
Moderately resistant	16.96 (2.17)	2.25 (1.89)	70.58 (7.88)	3.03 (0.68)	18.73 (2.83)
P	0,0982	0,0663	0,366	0,0517	0.0570

a Each reaction phenotype is represented by four isolates

b Reaction phenotype values for each variable do not differ significantly according to Student's t-test (P=0.05). Values (±) in parentheses represent standard errors

Capítulo IV

Conclusões Gerais

CONCLUSÕES GERAIS

- Diferentes espécies de *Colletotrichum* estão associadas à antracnose da banana no Brasil;

- Existe diferença entre os métodos automatizados de filtragem de alinhamentos quanto ao número de informatividade filogenética retida nos alinhamentos finais;

- A combinação dos marcadores moleculares APN2, APN2/MAT-IGS, CAL, GAPDH, GAP2-IGS, GS e TUB2 é eficiente para identificação de espécies pertencentes complexo *C. gloeosporioides*;

- Os primers Musae_tub_33F e Musae_tub_387 são eficientes para detecção rápida de isolados de *C. musae*;

- O meio de cultura interfere na variância dos dados das medições de conídios de *Colletotrichum*;

- Diferentes espécies de *Colletotrichum* diferem quanto ao nível de virulência em frutos de banana;

- Isolados de *C. musae* oriundos de diferentes plantios de banana do Brasil diferem quanto a sensibilidade ao tiofanato-metílico;

- O tiofanato-metílico, na sua dosagem comercial, não é eficaz no controle de isolados de *C. musae* moderadamente resistentes em frutos de bananeira;

- A mutação pontual no códon 200 confere a isolados de *C. musae* o caráter moderadamente-resistente ao tiofanato-metílico;

- Não há indicações de custo de adaptabilidade aos isolados de *C. musae* moderadamente resistentes.