# New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia

M. Van Wyk · B. D. Wingfield · M. Marin · M. J. Wingfield

**Abstract** *Ceratocystis fimbriata sensu lato* includes a large number of plant and especially tree pathogens. In Colombia, isolates of this fungus cause a serious canker-stain disease on coffee as well as other fruit trees. Large collections of these isolates have been shown to occur in two distinct phylogenetic lineages based on ITS sequence comparisons. The aim of this study was to compare representatives of these two groups of isolates from coffee, citrus, cacao and native trees in Colombia, based on morphology and DNA-sequences for three gene regions. Host-specificity of the fungus was also considered. Representatives of the two groups of isolates were morphologically distinct and could be distinguished based on DNA sequence comparisons. They are also distinct from other species in the *C. fimbriata sensu lato* species complex and the sweet potato pathogen *C. fimbriata sensu stricto* and are provided with the names *C. colombiana* sp. nov and *C. papillata* sp. nov. There was no evidence for host-specificity amongst isolates of these two fungi that collectively represent a serious threat to coffee production in Colombia.

**Keywords** Ophiostomatoid fungi · Phylogenetic relationships · Species concepts · Tree diseases

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## Introduction

Colombia is the world's largest producer of coffee (*Coffea arabica* L.) and depends on this product for foreign exchange. It is consequently worrying that this crop is seriously affected by a canker-stain disease caused by *Ceratocystis fimbriata* Ellis & Halst. *sensu lato* (*s.l.*). The first record of coffee canker disease caused by this fungus, was from the island of Java (Indonesia) in 1900 (Pontis 1951) and it was first recorded in Colombia in 1932 (Zimmerman 1900; Castaño 1951).

Canker stain disease caused by *C. fimbriata s.l.* in Colombia is found on a wide range of hosts other than coffee. These include citrus (*Citrus reticulata* Blanco, *Citrus sinensis* (L.) Osbeck and *Citrus limon* (L.) Burm. f.) barinas nut (*Caryodendron orinocense* H. Karst.), mango (*Mangifera indica* L.), rubber (*Hevea brasiliensis* (Willd.) Müll. Arg.) cacao (*Theobroma cacao* L.) and several native forest trees including *Schizolobium parahybum* (Vell.) S.F. Blake and *Annona muricata* L. (Castaño 1951; Webster and Butler 1967; Kile 1993; Mourichon 1994; Pardo-Cardona 1995; Marin et al. 2003). Coffee is frequently cultivated in close proximity to citrus and native trees and it is likely that inoculum of the pathogen is readily exchanged between these plants.

*Ceratocystis fimbriata s.l.* requires wounds for infection and these are created during pruning and other cultivation practices (Pontis 1951; Marin et al. 2003). In some cases, the source of infection is chlamydospore-infested soil (Rossetto and Ribeiro 1990; Marin et al. 2003; Marin 2004). *Ceratocystis fimbriata* was first described based on isolates of the fungus causing black rot of sweet potato in the United States of America (Halsted 1890). Subsequently, the fungus was associated with diseases of a large number of different plant species in many parts of the world. While

morphological differences between isolates of *C. fimbriata s.l.* were not clearly evident, Webster and Butler (1967) recognised that the fungus probably represented more than one entity. The availability of molecular techniques and particularly DNA sequence comparisons has dramatically changed the current taxonomic position of *C. fimbriata s.l.*

A contemporary view of *C. fimbriata s.l.* is that it represents a complex of many cryptic species. Thus, the sweet potato black rot pathogen is treated as *C. fimbriata sensu stricto* (*s.s.*). Numerous cryptic species that would previously have been treated as *C. fimbriata* have been described during the course of the last decade (Engelbrecht and Harrington 2005; Johnson et al. 2005; Van Wyk et al. 2004, 2006, 2007a, b). It also is likely that many other discrete and cryptic taxa reside amongst isolates of this fungus that have not been studied in detail.

Barnes et al. (2001) undertook the first DNA-based study on isolates in the *C. fimbriata s.l.* complex including isolates of *C. fimbriata s.l.* from coffee in Colombia. DNA sequence comparisons and analyses using microsatellite markers showed that the isolates from Colombia resided in two very distinct phylogenetic lineages (Barnes et al. 2001). Marin et al. (2003) compared 50 isolates of *C. fimbriata s.l.* from 11 different provinces in Colombia using DNA sequence data, RAPDs, RFLPs and pathogenicity tests. Their results also showed conclusively that isolates from Colombia represented two distinct entities that were highly variable.

The aim of this study was to compare isolates representing the two groups (Marin et al. 2003) encompassing *C. fimbriata s.l.* from Colombia. Comparisons of isolates were based on morphology and on sequences of three gene regions. Because isolates representing the two phylogenetic assemblages originated from different hosts, their ability to infect these trees was also considered using pathogenicity tests.

## Materials and methods

### Isolates

Isolates used in this study were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). These were also all used in the previous study of Marin et al. (2003). The isolates were selected based on host, geographic occurrence and groupings emerging from the studies of Barnes et al. (2001) and Marin et al. (2003).

### PCR and sequencing reactions

DNA was extracted from isolates as described by Van Wyk et al. (2006). Three gene regions were chosen for comparisons. These were the ITS 1 and 4 regions including the 5.8S gene of the rDNA operon, part of the $\beta$-tubulin gene and a portion of the Transcription Elongation Factor 1-$\alpha$ gene. The primers used for these reactions were those developed by White et al. (1990), Glass and Donaldson (1995) and Jacobs et al. (2004), respectively.

The PCR and sequencing conditions and reactions were as described by Van Wyk et al. (2006). Sequences for other closely related *Ceratocystis* species were obtained from previous studies (Van Wyk et al. 2004, 2007a, b, 2009) and analysed using PAUP version 4.0b10* (Swofford 2002). Sequences were aligned with the programme MAFFT (Katoh et al. 2002) and manually confirmed. A partition homogeneity test (PHT) (Swofford 2002) was used to determine whether the three datasets could be combined. Gaps were treated as a fifth character and the trees were obtained by the stepwise addition of 1,000 replicates with the Mulpar option in effect. The phylogram was obtained by selecting the heuristic search option with stepwise addition. Confidence intervals using 1,000 bootstrap replicates were calculated. Two isolates of *C. virescens* (R.W. Davidson) C. Moreau were designated to represent the outgroup and all sequences derived from this study were deposited in GenBank (Table 1).

The Markov Chain Monte Carlo (MCMC) algorithm was used to create a phylogenetic tree based on Bayesian probabilities using the software program MrBayes version 3.1.1 (Ronquist and Huelsenbeck 2003). For each of the three genes, a model of nucleotide substitution was determined using MrModeltest 2.2 (Nylander 2004). The nucleotide substitutions obtained were included for each gene partition in MrBayes. One million random trees were generated using the MCMC procedure with four chains and sampled every 100th generation. To avoid including trees that had been sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before the stabilization. Trees outside the point of convergence were discarded by means of the burn-in procedure in MrBayes (Ronquist and Huelsenbeck 2003).

The two groups of isolates from Colombia as well as *C. neglecta*, previously described from *Eucalyptus* in Colombia (Rodas et al. 2007), were analyzed separately for each of the three gene regions mentioned above. The PAUP settings were the same as those for the combined dataset, but without an outgroup. Molecular Evolutionary Genetics Analysis (MEGA) 4 (Tamura et al. 2007) was utilized to determine the level of variation between the isolates from Colombia including *C. neglecta*. Sequences for each gene region were examined to determine the number of fixed alleles between them. The software package TCS (Clement et al. 2000) was used to construct an allele network to observe the shared alleles between isolates representing the two groups from Colombia and the Colombian species *C. neglecta*.

**Table 1** Isolates of *Ceratocystis* used in this study

| Species | Isolate no. | GenBank accession no. | Host | Geographic origin |
|---------|-------------|----------------------|------|-------------------|
| *C. albifundus* | CMW4068 | DQ520638<br>EF070429<br>EF070400 | *Acacia mearnsii* | RSA |
| *C. albifundus* | CMW5329<br>CBS119681 | AF388947<br>DQ371649<br>EF070401 | *Acacia mearnsii* | Uganda |
| *C. atrox* | CMW19383<br>CBS120517 | EF070414<br>EF070430<br>EF070402 | *Eucalyptus grandis* | Australia |
| *C. atrox* | CMW19385<br>CBS120518 | EF070415<br>EF070431<br>EF070403 | *Eucalyptus grandis* | Australia |
| *C. cacaofunesta* | CMW15051<br>CBS152.62 | DQ520636<br>EF070427<br>EF070398 | *Theobroma cacao* | Costa Rica |
| *C. cacaofunesta* | CMW14809<br>CBS115169 | DQ520637<br>EF070428<br>EF070399 | *Theobroma cacao* | Ecuador |
| *C. caryae* | CMW14793<br>CBS114716 | EF070424<br>EF070439<br>EF070412 | *Carya cordiformis* | USA |
| *C. caryae* | CMW14808<br>CBS115168 | EF070423<br>EF070440<br>EF070411 | *Carya ovata* | USA |
| **C. colombiana** | **CMW9565**<br>**CBS121790** | **AY233864**<br>**AY233870**<br>**EU241487** | **Soil in coffee plantation** | **Colombia** |
| **C. colombiana** | **CMW9572**<br>**CBS123011** | **AY233863**<br>**AY233871**<br>**EU241488** | **Mandarin** | **Colombia** |
| **C. colombiana** | **CMW5768** | **AY177235**<br>**AY177222**<br>**EU241491** | **Coffea arabica** | **Colombia** |
| **C. colombiana** | **CMW5761**<br>**CBS121791** | **AY177234**<br>**AY177224**<br>**EU241492** | **Coffea arabica** | **Colombia** |
| **C. colombiana** | **CMW5751**<br>**CBS121792** | **AY177233**<br>**AY177225**<br>**EU241493** | **Coffea arabica** | **Colombia** |
| **C. colombiana** | **CMW11280** | **AY233861**<br>**AY233873**<br>**EU241490** | **Schizolobium parahybum** | **Colombia** |
| **C. colombiana** | **CMW10871** | **AY233862**<br>**AY233872**<br>**EU241489** | **Citrus sinensis** | **Colombia** |
| *C. fimbriata s.s.* | CMW15049<br>CBS141.37 | DQ520629<br>EF070442<br>EF070394 | *Ipomoea batatas* | USA |
| *C. fimbriata s.s.* | CMW1547<br>CBS123010 | AF264904<br>EF070443<br>EF070395 | *Ipomoea batatas* | Papua New Guinea |

**Table 1** (continued)

| Species | Isolate no. | GenBank accession no. | Host | Geographic origin |
|---------|-------------|----------------------|------|-------------------|
| *C. fimbriatomima* | CMW24174<br>CBS121786 | EF190963<br>EF190951<br>EF190957 | *Eucalyptus* sp. | Venezuela |
| *C. fimbriatomima* | CMW24176<br>CBS121787 | EF190964<br>EF190952<br>EF190958 | *Eucalyptus* sp. | Venezuela |
| *C. manginecans* | CMW13851<br>CBS121659 | AY953383<br>EF433308<br>EF433317 | *Mangifera indica* | Oman |
| *C. manginecans* | CMW13852<br>CBS121660 | AY953384<br>EF433309<br>EF433318 | *Mangifera indica* | Oman |
| *C. manginecans* | CMW13854 | AY953385<br>EF433310<br>EF433319 | *Mangifera indica* | Oman |
| *C. neglecta* | CMW17808<br>CBS121789 | EF127990<br>EU881898<br>EU881904 | *Eucalyptus* | Colombia |
| *C. neglecta* | CMW18194<br>CBS121017 | EF127991<br>EU881899<br>EU881905 | *Eucalyptus* | Colombia |
| **C. papillata** | **CMW10844** | **AY177238**<br>**AY177229**<br>**EU241481** | **Coffea arabica** | **Colombia** |
| **C. papillata** | **CMW5746** | **EU241479**<br>**EU241480**<br>**EU241482** | **Coffea arabica** | **Colombia** |
| **C. papillata** | **CMW8857** | **AY233868**<br>**AY233878**<br>**EU241483** | **Annona muricata** | **Colombia** |
| **C. papillata** | **CMW8856**<br>**CBS121793** | **AY233867**<br>**AY233874**<br>**EU241484** | **Citrus limon** | **Colombia** |
| **C. papillata** | **CMW 8850**<br>**CBS121794** | **AY233866**<br>**AY233875**<br>**EU241485** | **Citrus x tangelo** | **Colombia** |
| **C. papillata** | **CMW 8858**<br>**CBS121795** | **AY233865**<br>**AY233877**<br>**EU241486** | **Schizolobium parahybum** | **Colombia** |
| **C. papillata** | **CMW8860** | **GQ478239**<br>**GQ478241**<br>**GQ478237** | **Theobroma cacao** | **Colombia** |
| **C. papillata** | **CMW9561** | **GQ478240**<br>**GQ478242**<br>**GQ478238** | **Theobroma cacao** | **Colombia** |
| *C. pirilliformis* | CMW6569 | AF427104<br>DQ371652<br>AY528982 | *Eucalyptus nitens* | Australia |
| *C. pirilliformis* | CMW6579<br>CBS118128 | AF427105<br>DQ371653<br>AY528983 | *Eucalyptus nitens* | Australia |
| *C. platani* | CMW14802 | DQ520630 | *Platanus occidentalis* | USA |

**Table 1** (continued)

| Species | Isolate no. | GenBank accession no. | Host | Geographic origin |
|---|---|---|---|---|
| | CBS115162 | EF070425 | | |
| | | EF070396 | | |
| *C. platani* | CMW23918 | EF070426 | *Platanus occidentalis* | Greece |
| | | EF070397 | | |
| *C. polychroma* | CMW11424 | AY528970 | *Syzygium aromaticum* | Indonesia |
| | CBS115778 | AY528966 | | |
| | | AY528978 | | |
| *C. polychroma* | CMW11436 | AY528971 | *Syzygium aromaticum* | Indonesia |
| | CBS115777 | AY528967 | | |
| | | AY528979 | | |
| *C. populicola* | CMW14789 | EF070418 | *Populus* sp. | Poland |
| | CBS119.78 | EF070434 | | |
| | | EF070406 | | |
| *C. populicola* | CMW14819 | EF070419 | *Populus* sp. | USA |
| | CBS114725 | EF070435 | | |
| | | EF070407 | | |
| *C. smalleyi* | CMW26383 | EU426553 | *Carya cordiformis* | USA |
| | CBS114724 | EU426555 | | |
| | | EU426556 | | |
| *C. smalleyi* | CMW14800 | EF070420 | *Carya cordiformis* | USA |
| | CBS114724 | EF070436 | | |
| | | EF070408 | | |
| *C. tanganyicensis* | CMW15991 | EU244997 | *Acacia mearnsii* | Tanzania |
| | CBS122295 | EU244969 | | |
| | | EU244929 | | |
| *C. tanganyicensis* | CMW15999 | EU244998 | *Acacia mearnsii* | Tanzania |
| | CBS122294 | EU244970 | | |
| | | EU244939 | | |
| *C. tsitsikammensis* | CMW14276 | EF408555 | *Rapanea melanophloeos* | South Africa |
| | CBS121018 | EF408569 | | |
| | | EF408576 | | |
| *C. tsitsikammensis* | CMW14278 | EF408556 | *Rapanea melanophloeos* | South Africa |
| | CBS121019 | EF408570 | | |
| | | EF408577 | | |
| *C. variospora* | CMW20935 | EF070421 | *Quercus alba* | USA |
| | CBS114715 | EF070437 | | |
| | | EF070409 | | |
| *C. variospora* | CMW20936 | EF070422 | *Quercus robur* | USA |
| | CBS114714 | EF070438 | | |
| | | EF070410 | | |
| *C. virescens* | CMW11164 | DQ520639 | *Fagus americanum* | USA |
| | CBS123166 | EF070441 | | |
| | | EF070413 | | |
| *C. virescens* | CMW3276 | AY528984 | *Quercus robur* | USA |
| | CBS123216 | AY528990 | | |
| | | AY529011 | | |

Bold face indicate the cultures used in this study

Culture characteristics and morphology

Three isolates representing each of the two groups identified by Marin et al. (2003) (CMW5751, CMW5761, CMW10871 and CMW8856, CMW8857, CMW8850) were selected for morphological studies. Colony colour was determined using the colour tables of Rayner (1970). A growth study was conducted to determine the temperatures for optimal growth of the two groups of isolates. Cultures were grown on 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with Streptomycin Sulphate (0.001 g vol$^{-1}$, SIGMA, Steinheim, Germany) for 2 weeks. A 4 mm plug was taken from the edges of actively growing cultures and placed at the centres of 90 mm Petri dishes containing 2% MEA. Five replicate plates for each isolate were incubated at temperatures ranging form 5°C to 35°C at 5°C intervals. Growth was assessed by measuring colony diameters after 7 days. The average growth and standard deviations were then calculated. The entire experiment was repeated once.

The same six isolates, representing the two groups, used in the growth studies were used for morphological examinations. Fungal structures were mounted in lactic acid on glass slides and examined using a Carl Zeiss microscope and the photographic images were captured with a Zeiss Axio Vision camera system. Fifty measurements were taken for each taxonomically relevant structure in isolates CMW5751 and CMW8856, which were chosen to represent the two groups respectively. In addition, ten measurements were made of the relevant structures for the four remaining isolates representing the two groups. Ranges, averages, and standard deviations of all measurements were calculated and measurements are presented as (minimum-) mean minus standard deviation minus—mean plus standard deviation (-maximum-).

Reciprocal inoculation tests

In order to test differences in pathogenicity amongst isolates obtained from coffee, cacao and citrus (Valencia orange), a greenhouse inoculation experiment was conducted in Chinchiná, Colombia. Four randomly chosen isolates from coffee, two from each group (Marin et al. 2003), two isolates from cacao and two isolates from citrus, were used. These eight strains were inoculated into the stems of nine-month-old plants of coffee variety Caturra, eight-month-old plants of cacao variety IMC-67 and 12-month-old plants of Valencia orange.

A cambial disc was removed from the main stems of 40 plants using a 5-mm-diameter cork borer. A similar sized plug taken from the actively growing margins of cultures of each isolate growing on 2% MEA plates were placed in the wounds and covered with Parafilm to reduce desiccation

and contamination. A set of 40 plants of each host was inoculated in a similar way, but using sterile MEA discs as a control treatment. After 21 weeks, the lengths of necrotic lesions in the phloem (coffee) or the vertical extension of discoloration in the xylem (cacao and citrus) was determined. Data were analysed using one-way analysis of variance (ANOVA) for each host. Tukey's HSD multiple comparisons were performed to test for statistically significant differences amongst isolates (SYSTAT, SPSS Inc., Chicago).
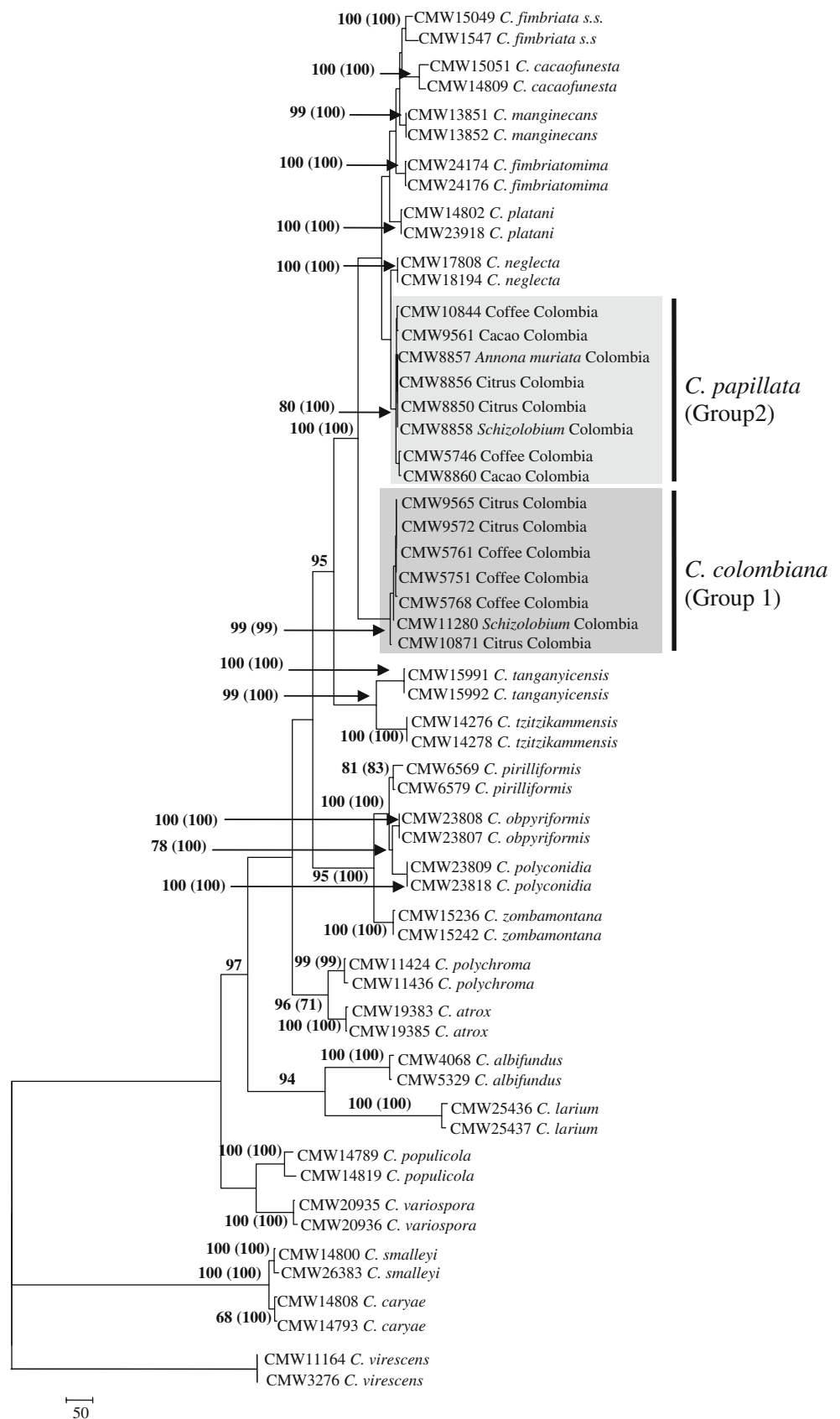
Results

PCR and sequence analyses

Amplicons of ~500 bp were obtained for the ITS and $\beta$-tubulin regions and a ~800 bp fragment was obtained for the Transcription Elongation Factor 1-$\alpha$ region. The PHT gave a value of $P=0.01$, which allowed for the datasets being combined (Cunningham 1997). The dataset consisted of 1,971 characters, 1,104 were constant characters, 46 were parsimony uninformative and 821 were parsimony informative. Four most parsimonious trees were obtained from this dataset, one of which one was selected for presentation (Fig. 1). This tree had a length of 2205, a consistency index of 0.7, a retention index of 0.9 and a rescaled consistency index of 0.6.

For the dataset representing the ITS region, the model obtained with MrModeltest2 that was used in the Bayesian analysis was the GTR+I+G model. For the partial $\beta$-tubulin gene dataset, the GTR+G model was selected while for the EF1-$\alpha$ gene dataset, the HKY+I+G model was selected. Two thousand trees were discarded due to the fact that they were outside the point of convergence (burn-in). Bayesian inference used to obtain the posterior probability of the branch nodes of the combined tree supported the bootstrap values obtained with PAUP (100% and 100%, respectively) (Fig. 1).

In the phylogenetic tree (Fig. 1), two groups of isolates from Colombia were phylogenetically distinct from each other. The groups had a high bootstrap support of 99% and 80%, respectively. Isolates in both groups showed a high level of variation in sequences and both groups included isolates from native trees, citrus, cacao and coffee. The species most closely related to those representing the two groups from Colombia were *C. fimbriata s.s.*, *C. cacaofunesta* Engelbr. & T.C. Harr., *C. platani* Englbr. & T.C. Harr., *C. fimbriatomima* M. van Wyk & M.J. Wingf, *C. neglecta* M. van Wyk, Jol. Roux & Rodas and *C. manginecans* M. van Wyk, Al Adawi, M.J. Wingf.

Phylogenetic trees derived from sequences for the single gene regions (ITS, βT and EF1-$\alpha$, respectively), separated the two groups of isolates from each other and from *C.*

**Fig. 1** Most parsimonious phylogenetic tree representing isolates in the *C. fimbriata s.l.* species complex and based on combined sequence data for the ITS, βT and EF1-α gene regions. The two distinct groups representing isolates from Colombia (Group 1 and Group 2) represent the new species *C. papillata* and *C. colombiana*. The dataset consisted of 1,971 characters, 46 parsimony uninformative characters, 821 parsimony informative characters. Tree length was 2205, consistency index = 0.7, retention index = 0.9 and rescaled consistency index = 0.6
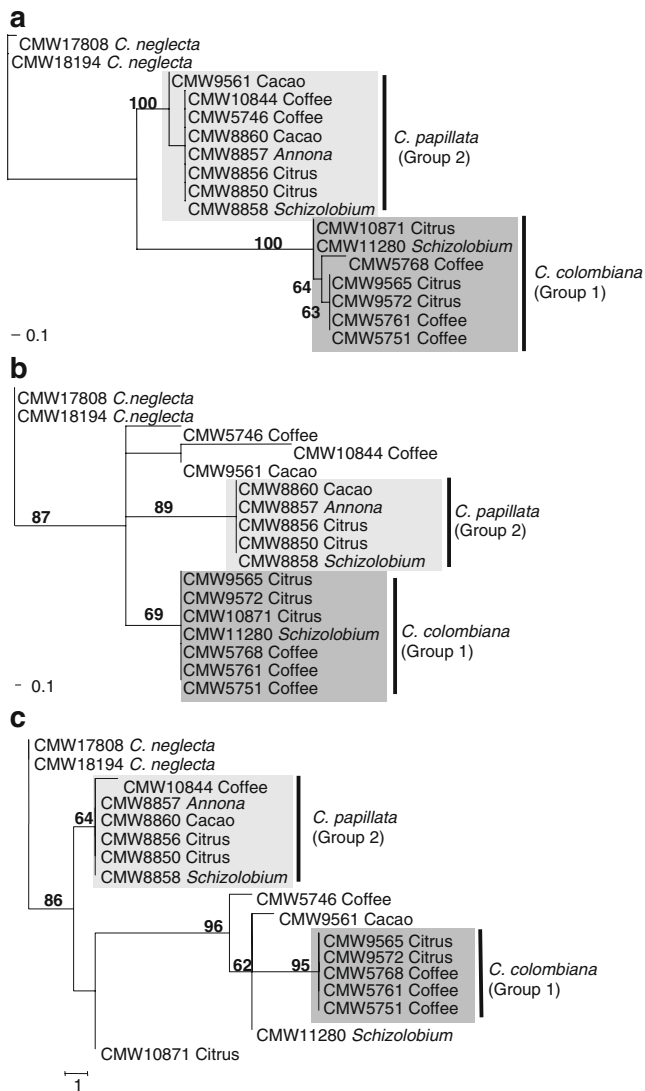
100 (100) CMW15049 *C. fimbriata s.s.*
CMW1547 *C. fimbriata s.s*

100 (100) CMW15051 *C. cacaofunesta*
CMW14809 *C. cacaofunesta*

99 (100) CMW13851 *C. manginecans*
CMW13852 *C. manginecans*

100 (100) CMW24174 *C. fimbriatomima*
CMW24176 *C. fimbriatomima*

100 (100) CMW14802 *C. platani*
CMW23918 *C. platani*

100 (100) CMW17808 *C. neglecta*
CMW18194 *C. neglecta*

80 (100)
100 (100)
CMW10844 Coffee Colombia
CMW9561 Cacao Colombia
CMW8857 *Annona muriata* Colombia
CMW8856 Citrus Colombia
CMW8850 Citrus Colombia
CMW8858 *Schizolobium* Colombia
CMW5746 Coffee Colombia
CMW8860 Cacao Colombia

*C. papillata* (Group2)

95
CMW9565 Citrus Colombia
CMW9572 Citrus Colombia
CMW5761 Coffee Colombia
CMW5751 Coffee Colombia
CMW5768 Coffee Colombia
99 (99) CMW11280 *Schizolobium* Colombia
CMW10871 Citrus Colombia

*C. colombiana* (Group 1)

100 (100) CMW15991 *C. tanganyicensis*
99 (100) CMW15992 *C. tanganyicensis*

100 (100) CMW14276 *C. tzitzikammensis*
CMW14278 *C. tzitzikammensis*

81 (83) CMW6569 *C. pirilliformis*
CMW6579 *C. pirilliformis*

100 (100) CMW23808 *C. obpyriformis*
100 (100) 78 (100) CMW23807 *C. obpyriformis*

CMW23809 *C. polyconidia*
95 (100) CMW23818 *C. polyconidia*
100 (100)

100 (100) CMW15236 *C. zombamontana*
CMW15242 *C. zombamontana*

99 (99) CMW11424 *C. polychroma*
CMW11436 *C. polychroma*

97
96 (71) CMW19383 *C. atrox*
100 (100) CMW19385 *C. atrox*

100 (100) CMW4068 *C. albifundus*
94 CMW5329 *C. albifundus*

100 (100) CMW25436 *C. larium*
CMW25437 *C. larium*

100 (100) CMW14789 *C. populicola*
CMW14819 *C. populicola*

CMW20935 *C. variospora*
100 (100) CMW20936 *C. variospora*

100 (100) CMW14800 *C. smalleyi*
100 (100) CMW26383 *C. smalleyi*

CMW14808 *C. caryae*
68 (100) CMW14793 *C. caryae*

CMW11164 *C. virescens*
CMW3276 *C. virescens*

50

Fig. 2 Three separate unrooted phylogenetic trees representing the **a** ITS, **b** the βT and the **c** EF1-α gene regions for isolates representing the two groups (Group 1 = *C. colombiana*; Group 2 = *C. papillata*) from Colombia and *C. neglecta*. The two groups of isolates from Colombia were separated from each other and from *C. neglecta* and had structures very similar to that for combined dataset. For the ITS tree, the bootstrap support was high for both groups of isolates. For the βT tree the bootstrap support was high for *C. papillata* and lower for *C. colombiana* and there were some outlying isolates, however, the structure remained the same as the combined tree. For the EF1-α tree there was support for separation of *C. colombiana* and *C. papillata* but there were some outlying isolates

*neglecta* and had structures (Fig. 2) very similar to that for the tree (Fig. 1) representing the combined data set for the three gene regions. However, the suport for the branches separating the groups was not as high as that found in the combined dataset.

Fixed polymorphisms between isolates representing the two groups from Colombia and *C. neglecta* were observed for all three gene regions. For the ITS region, there were 16

differences between the two groups of isolates from Colombia. Likewise, there were 19 fixed polymorphisms separating *C. neglecta* and Group 1 isolates and six differences between *C. neglecta* and Group 2 isolates in the ITS region. For the βT region there was one fixed allele separating the two groups of isolates form Colombia. Likewise, there were three fixed alleles separating *C. neglecta* and Group 1 isolates and two fixed alleles separating *C. neglecta* and Group 2 isolates. In the EF1-α region there were no fixed alleles separating the two groups of isolates from Colombia but there were two fixed alleles separating both *C. neglecta* and Group 1 and the same was true for *C. neglecta* and Group 2 isolates. The allele networks (Fig. 3) drawn to show genetic linkage between the two groups of isolates from Colombia or between those groups and *C. neglecta* showed no linkage between these three groups.
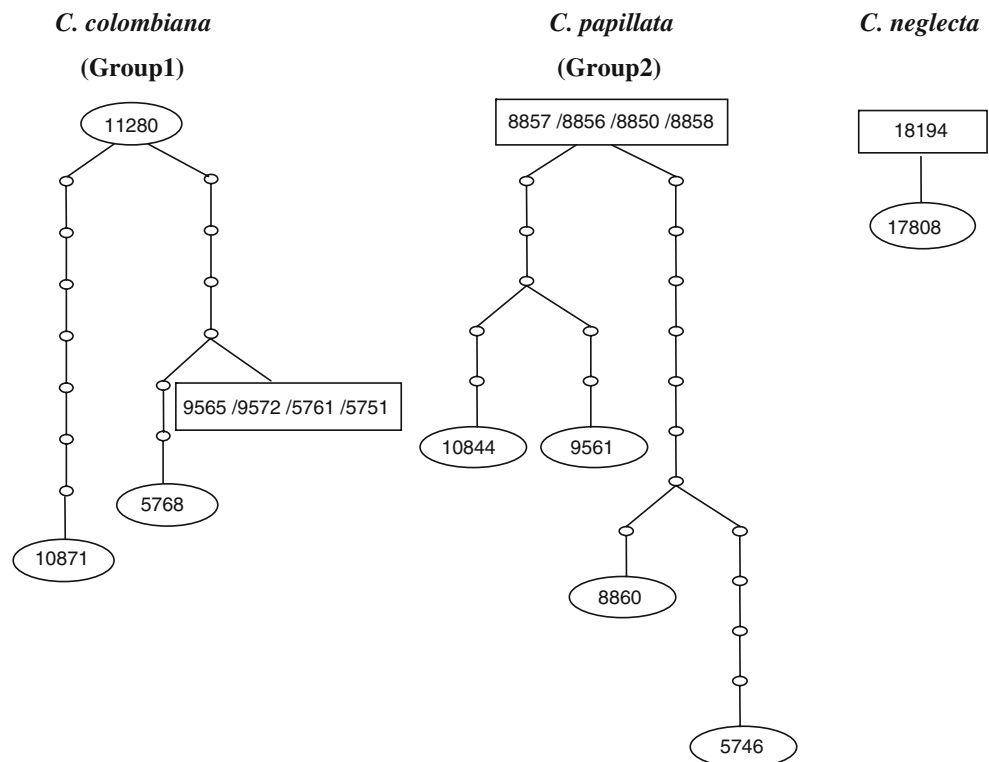
Culture characteristics and morphology

Cultures representing isolates occurring in the two groups from Colombian *C. fimbriata s.l.* clades were morphologically different. Isolates (CMW5751, CMW5761, CMW10871) representing Group 1 had a stellate appearance with ascomata abundant in the cultures that were a light brownish olive (19″k) colour. Group 2 isolates (CMW8856, CMW8857, CMW8850) displayed a deep greyish olive colour (21‴i).

Growth comparisons in culture showed that isolates (CMW8856, CMW 8857, CMW8850 ) representing Group 2 grew faster than isolates (CMW5751, CMW5761, CMW10871) in Group 1 at 15°C, 20°C, 25°C and 30°C. Neither group of isolates showed growth at 5°C, 10°C and 35°C. The optimum temperature for growth of isolates in Group 1 was 25°C with the cultures having an average diameter of 32 mm in 7 days. Group 2 isolates also grew optimally at 25°C attaining an average diameter of 31 mm (20°C), 36 mm (25°C) and 23 mm (30°C) respectively in 7 days.

Reciprocal inoculation tests

After 21 weeks, all isolates, irrespective of the host plant of origin, produced lesions on the stems of inoculated coffee plants. Seven of the isolates gave rise to necrotic lesions in the phloem ranging on an average from 50 to 106 mm long (Fig. 4a). These plants also developed chlorotic foliage and epicormic shoots below the inoculation points. Isolate CMW9561 from cacao caused lesions that were on average only 21 mm long, while the other isolate from cacao gave rise to lesions with an average length similar to those associated with the coffee isolates. Statistical analyses indicated significant differences in the lengths of lesions in the

**Fig. 3** Three allele networks derived from a single dataset of all three gene regions representing *C. colombiana*, *C. papillata* and *C. neglecta*. All numbers in allele network represent CMW numbers that are listed in Table 1. Within the *C. colombiana* network, there was high variation and up to 6 differences between some isolates. Within the *C. papillata* network there were also high levels of variation with up to 11 differences between some isolates. In both *C. colombiana* and *C. papillata* there was a single genotype that was dominant

phloem caused by the isolates (Fig. 4a). Isolate CMW8850 from Valencia orange was the most pathogenic to coffee plants and gave rise to an average lesion length of 106 mm. Isolates residing in the two groups from coffee did not produce significantly different lesion lengths on that host.

Extensive xylem discoloration was produced by all isolates inoculated onto cacao. The average lengths of internal discoloration ranged from 173 mm (CMW5746 from coffee) to 264 mm (CMW9561 from cacao) (Fig. 4b). Isolate CMW9561 from cacao produced the longest average lesion lengths, but no significant differences were detected between this isolate and other isolates. The high level of susceptibility of this host originating from isolates obtained from different hosts was evident from the very long individual lesions on some plants, for example 650 mm (CMW5761 from coffee), 620 mm (CMW8860 from cacao) and 470 mm (CMW10844 from coffee) (Fig. 4b).

Xylem discoloration on Valencia orange plants ranged from 53 mm (CMW9561 from cacao) to 193 mm (CMW5761 from coffee). Five isolates (CMW10844 from coffee, CMW5746 from coffee, CMW8850 from citrus, CMW8860 from cacao and CMW5751 from coffee) recovered from different hosts produced statistically indistinguishable lengths of discoloration (Fig. 4c). Citrus isolates were moderately pathogenic, while isolate CMW5761 from coffee was most pathogenic on this host (Fig. 4c). Control inoculations did not give rise to lesions on any of the hosts tested (Fig. 4a–c).

## Taxonomy

The *C. fimbriata s.l.* isolates from Colombia considered in this study clearly represent two unique taxa. These differ from each other based on morphology and DNA sequence comparisons for three gene regions. They also differ from all described species of *Ceratocystis* based on morphological characteristics and they are thus described as follows:

***Ceratocystis colombiana*** M. van Wyk & M.J. Wingf., **sp. nov.**

(Fig 5a–h)

Mycobank: 511243.

Etymology—Name refers to the Colombian origin of this fungus.

Ascomata typica generis, atrobrunnea vel nigra, collo apicem versus pallescenti. Bases peritheciorum globosae. Colla ascomatum, apice cum hyphis ostiolaribus divergentibus. Ascosporae pileiformes. Conidiophorae biformes; phialides primariae ampulliformes apicem versus contractae. Conidia primaria cylindrica. Conidiophorae secondariae apice subinfundibuliformes. Conidia secondaria doliiformia Chlamydosporae coffeinae vel umbrinae.

*Culture morphology* stellate, ascomata abundant, light brownish olive (19″k). *Ascomata* dark-brown to black with the necks becoming lighter towards the apices. *Ascomatal bases* globose (140–)177–237(–294) μm diam. *Ascomatal necks* (375–)448–560(–676) μm long, (24–)27–35(–43) μm wide at base, (12–)14–18(–19) μm wide at apices.
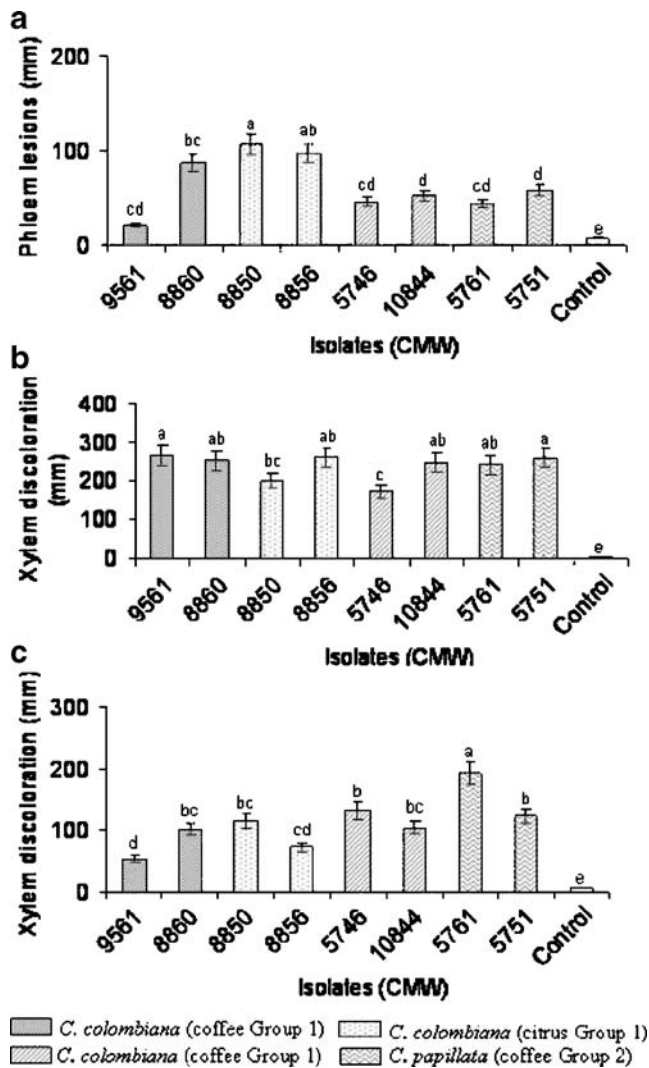
Fig. 4 Mean lesion lengths (±SEM) in the phloem after inoculation with eight isolates representing *C. colombiana* and *C. papillata* obtained from coffee (CMW5761, CMW5751; CMW10844, CMW5746), cacao (CMW8860, CMW9561) and citrus (CMW8851, CMW8852). **a** Inoculations on nine-month-old coffee plants (variety Caturra). **b** Inoculation on eight-month-old cacao plants (variety IMC-67). **c** Inoculation on twelve-month-old Valencia orange plants. *Bars* in the histogram annotated with different letters differ significantly from each other Isolates followed by different letters differ significantly from each other in terms of lesion lengths (*P*<0.05)

Ascomatal necks terminating in divergent *ostiolar hyphae*, (28–)38–46(–52) μm long. *Ascospores* hat-shaped 3–4 μm long, (3–)4–6(–7) μm wide excluding sheaths, 6–8(–11) μm including sheaths.

*Conidiophores* of two types. *Primary conidiophores* flask-shaped phialides tapering towards the apices. *Primary phialides* (58–)65–83(–106) μm long, 4–6(–8) μm wide at base, (3–)6–8(–9) μm wide at the broadest point and 3–5(–6) μm wide at apices. *Primary conidia*, cylindrical, (12–)16–24 (–29) μm long and 4–6 μm wide. *Secondary conidiophores*

flaring at apices. *Secondary phialides* (42–)49–71(–85) μm long, (4–)5–7 μm wide at the base, (5–)6–8 μm wide at apices. *Secondary conidia*, barrel-shaped, 9–14 μm long, 6–8(–11) μm wide. *Chlamydospores* hair-brown, globose, 11–14 μm long, 11–15(–17) μm wide.

*Habitat*: *Coffea arabica*, soil, citrus trees, *Schizolobium parahybum*.

*Known distribution*: Colombia, South America.

*Material examined*. COLOMBIA, Valle del Cauca, from diseased *Coffea arabica* trees, *M. Marin*, **holotype** Herb. PREM 59434, culture ex-type CMW 5751 = CBS 121792, 2000. COLOMBIA, *Coffea arabica* trees, *M. Marin*, PREM 59435, culture CMW 5761 = CBS 121791, 2000. COLOMBIA, Caldas, from soil in citrus orchard, *B. Castro* PREM 59619, culture CMW9565 = CBS 121790, 2002. COLOMBIA, Caldas, from soil in citrus (Tangelo) orchard, *B. Castro* PREM 59619, culture CMW 9565. COLOMBIA, Caldas, Mandarin, *B. Castro* PREM 60177, culture CMW 9572, 2002. COLOMBIA, Caldas, *C. arabica*, *M. Marin* PREM 60175, culture CMW 5768, 2000. COLOMBIA, Santander, *Schizolobium parahybum*, *unknown*, PREM 60176, culture CMW 11280, 2001. COLOMBIA, Caldas, *Citrus sinensis* (Valencia orange), *M. Marin* PREM 60178, culture CMW 10871, 2001.

*Notes*: *Ceratocystis colombiana* can be distinguished from its closest phylogenetic relative by the presence of secondary conidiophores and the secondary barrel-shaped conidia.

**Ceratocystis papillata** M. van Wyk & M.J. Wingf., **sp. nov.**,
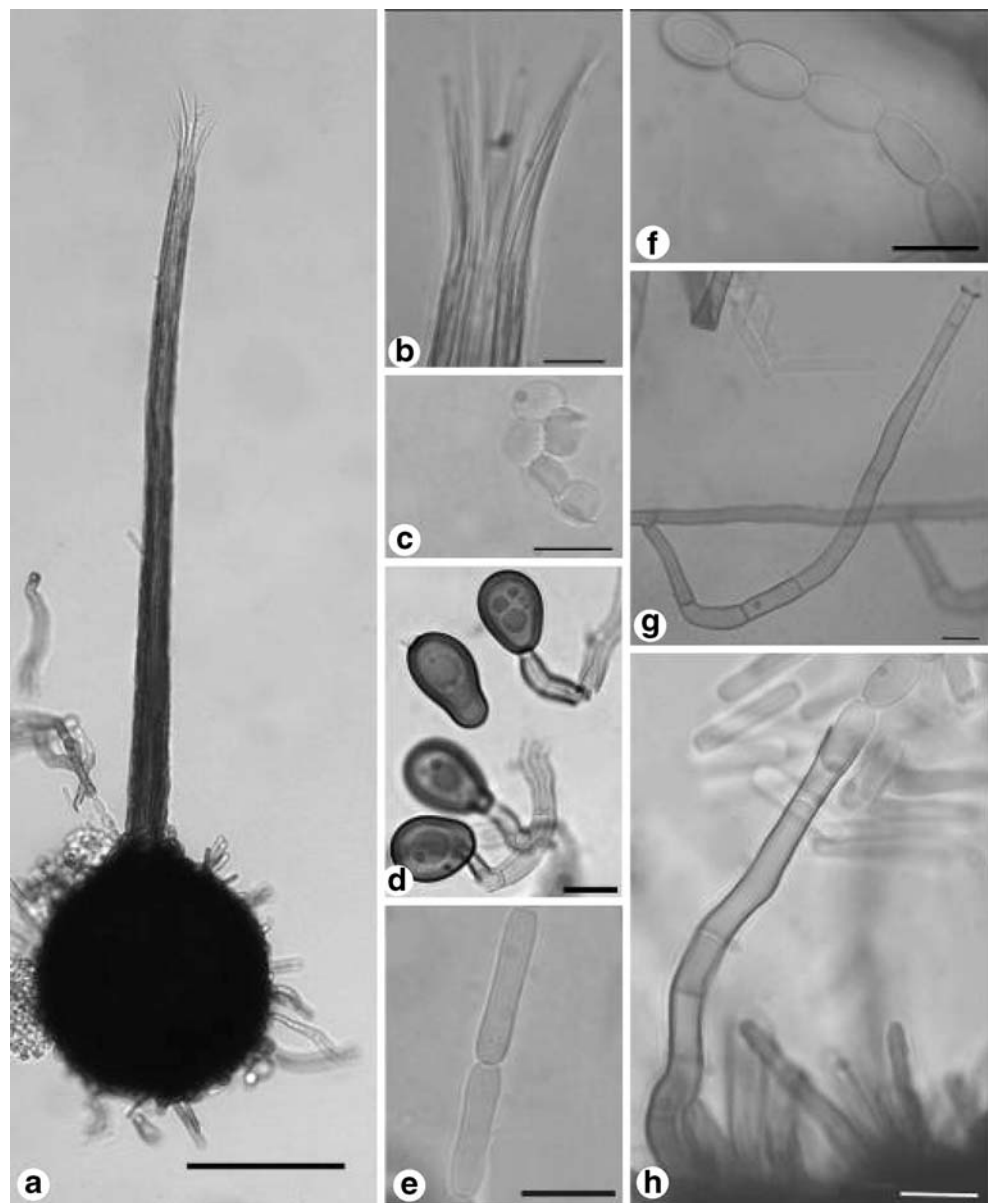
(Fig 6a–h)

Mycobank: 511244.

Etymology—Name refers to the papilla like form of the apices of the ascomatal bases

Ascomata atrobrunnea vel nigra, globosa collis elongatis apicem versus pallescentibus; bases globosae. Supra cum structura torulosa; colla. Hyphae ostiolares divergentes. Ascosporae pileiformes. Conidiophora primaria ampulliformis, apicem versus contracta. Conidia primaria cylindrica. Phialides secondariae tubiformes. Conidia secondaria ellipsoideo-doliiformia. Chlamydosporae subglobosae vel globosae, coffeinae vel umbrinae.

*Culture morphology* deep greyish olive in colour (21''''i). *Ascomata* dark-brown to black in colour, globose, necks long becoming lighter towards the apices. *Ascomatal bases* globose, with papillate apex, (160–)177–233(–258) μm diam. *Ascomatal necks* (472–)614–724(–753) μm long, (30–)33–47(–58) μm wide at base, (13–)17–21(–25) μm wide at apices. *Ostiolar hyphae* divergent, (44–)55–71(–78) μm long. *Ascospores* hat-shaped, 3–4 μm long, (3–)4–6 μm in width without sheath, 5–7(–8) μm wide including the sheath.

*Primary conidiophores* flask shaped, tapered towards the apices, (106–)116–164(–184) μm long, the bases are (3–)4–

Fig. 5 Morphological characteristics of isolate (CMW 5751) representing *C. colombiana*. **a** Ascoma with globose base and elongated neck terminating in divergent ostiolar hyphae. **b** Divergent ostiolar hyphae. **c** Hat-shaped ascospores. **d** Chlamydospores. **e** Cylindrical conidia. **f** Chain of barrel-shaped conidia. **g** Primary flask-shaped phialides. **h** Secondary phialides with flared apex. (*Scale bars*: A = 100 μm, B = 20 μm, C = 5 μm, D, F–H = 10 μm, E = 20 μm)



6(–7) μm wide, at the broadest point (6–)7–9 μm wide and at the apices 3–5(–6) μm wide. *Primary conidia* cylindrical, (17–)19–25(–29) μm long, (3–)4–6(–7) μm wide. *Secondary phialides* tubular, (46–) 48–66 (–76) μm long, bases 3–5 μm wide, apices 3–6 μm wide. *Secondary conidia* barrel-shaped, (6–)9–11 μm long, 5–7(–8) μm wide. *Chlamydospores* sub-globose to globose, hair-brown, (10–)12–14(–16) μm long, (8–)10–12(–14) μm wide.

*Habitat*: *Coffea arabica*, Soil, *Theobroma cacao*, *Schizolobium parahybum*, *Citrus x tangelo*, *Citrus limon*, *Annona muricata*.

*Known distribution*: Colombia, South America.

*Material examined*: COLOMBIA, Caldas, *Citrus x tangelo* B. Castro **holotype** Herb. PREM 59438, culture ex-type CMW 8856 = CBS 121793, 2001. COLOMBIA, Quindio, *Citrus limon*, B. Castro PREM 59620, CMW 8850 = CBS 121794, 2001. COLOMBIA, Quindio, *Schizolobium parahybum*, B. Castro **paratype** PREM 59621, CMW 8858 = CBS 121795, 2001. COLOMBIA, Risaralda, *Annona muricata*, B. Castro PREM 60173, CMW 8857, 2001. COLOMBIA, Caldas, *C. arabica*, M.J. Wingfield PREM 60171, CMW 5746, 2000. COLOMBIA, Antioquia, *C. arabica*, M. Marin, PREM 60172, CMW 10844, 1998. COLOMBIA, Caldas, *Theobroma cacao*, B. Castro, PREM 60174, CMW 8860, 2001. COLOMBIA, Caldas, *Theobroma cacao*, B. Castro, CMW 9561, 2001.

*Notes*: *Ceratocystis papillata* can be distinguished from most species in the *C. fimbriata s.l.* species complex by the presence of the cap-like morphology of the apices of the ascomatal bases. The only other species that have a similar ascomatal base morphology are *C. caryae*, *C. smalleyi*, *C. variospora* and *C. populicola* J.A. Johnson & T.C. Harr.,

**Fig. 6** Morphological characteristics of *C. papillata* (CMW 8856) from Colombia isolated from coffee trees. **a** Globose ascomata. **b** Divergent ostiolar hyphae. **c** Ascomatal base with papillate apex. **d** Hat-shaped ascospores. **e** Cylindrical and barrel-shaped conidia. **f** Chlamydospores. **g** Primary flasked-shaped phialide. **h** Secondary flaring phialide. (Scale A = 100 μm, B = 20 μm, C = 100 μm, D = 5 μm, E–H = 10 μm)

but there are clear differences between *C. papillata* and these species. These differences include absence of primary conidiophores in *C. smalleyi*, the smaller sizes of both conidiophores of *C. papillata* compared to those of *C. caryae*, the shorter necks of *C. populicola* and the larger ascomatal bases of *C. variospora*.

## Discussion

Mutligene phylogeny has proved useful in distinguishing species complexes in several genera of fungi including *Ceratocystis* (Van Wyk et al. 2004, 2007a, b, 2009), *Colletotrichum* (Yang et al. 2009; Prihastuti et al. 2009), *Diplodia* and *Lasiodiplodia* (Lazzizera et al. 2008; Alves et al. 2008), *Fusarium* (Kvas et al. 2009), *Mycosphaerella*

(Crous 2009) and *Phomopsis* (Santos and Phillips 2009) and has also revealed two cryptic species in this study. Previous studies have shown that isolates of *C. fimbriata s.l.* from coffee in Colombia reside in two distinct phylogenetic lineages (Barnes et al. 2001; Marin et al. 2003). Results of the present investigation confirm those results and they have shown that the species in the two groups differ phylogenetically based on sequences for three gene regions, from all described species in the *C. fimbriata s.l.* species complex. Fixed polymorphisms were found in two of the three gene regions between representatives of the two groups of isolates and in three gene regions between these fungi and *C. neglecta*, which is a closely related species also from Colombia. Allele networks based on sequence data for three gene regions also showed robust separation between isolates representing

the two groups of isolates from Colombia. Furthermore, isolates residing in the two phylogenetic lineages from Colombia were morphologically distinct from each other. These results support the conclusion that isolates representing the two phylogenetic groups of isolates from Colombia represent novel taxa for which the names *C. papillata* and *C. colombiana* have been provided.

Isolates of *C. papillata* and *C. colombiana* reside in robust phylogenetic clades distinct from all other species in the *C. fimbriata s.l.* species complex with high bootstrap and Bayesian support. There was considerable variation in the sequences of the individual isolates considered. These are clearly species with high genetic variability and are most likely native to the area in which they were collected. Sequence variation might also suggest that these fungi could represent species complexes that cannot be resolved at the present time. Single gene trees gave the same phylogenetic separation of isolates as that derived for the combined dataset, although a few isolates did not have strong support for their final grouping. The species phylogenetically most closely related to *C. papillata* and *C. colombiana* are *C. fimbriata s.s.*, *C. platani*, *C. manginecans*, *C. neglecta*, *C. fimbriatomima* and *C. cacaofunesta*.

*Ceratocystis papillata* can be distinguished from *C. colombiana* based on differences in culture morphology on 2% MEA. The stellate appearance of *C. colombiana* cultures is very distinct and makes it possible to easily distinguish between the two species. They also differ in their growth optima in culture and in various morphological characteristics. The most obvious morphological difference between the two species is found in the fact that isolates of *C. papillata* have ascomatal bases that have papillate apices. These are very distinct from the globose bases of *C. colombiana*. *Ceratocystis papillata* also has much longer ostiolar hyphae and the ascospore sheaths are much shorter than those of *C. colombiana*. Furthermore, the primary phialides of *C. papillata*, which are most commonly encountered, are double the length of those of *C. colombiana*. The barrel-shaped conidia in *C. papillata* were also substantially smaller than those of *C. colombiana*.

*Ceratocystis papillata* is morphologically similar to other species in the *C. fimbriata s.l.* species complex. The most distinct difference between this and other species is the characteristic "cap-like" morphology of the apices of the ascomatal bases in *C. papillata*. Furthermore, *C. papillata* can be distinguished from *C. fimbriata s.s.* by the absence of secondary conidiophores and secondary conidia, both of which are present in *C. papillata*. Other differences are the longer ascomatal necks and ostiolar hyphae of *C. fimbriata s.s.* when compared with *C. papillata*. The two species can also be separated by the shorter primary conidiophores of *C. fimbriata s.s.*, compared with those of *C. papillata*.

The cap-like apices of the ascomatal bases of *C. papillata* have been observed in other species in the *C. fimbriata s.l.* species complex, e.g. *C. caryae*, *C. smalleyi*, *C. variospora* and *C. populicola* (Johnson et al. 2005). However, this morphological feature is always present in *C. papillata* while it is only occasionally found in *C. caryae* (Johnson et al. 2005). This morphology in *C. caryae* was described as a part of the ascomatal neck (Johnson et al. 2005) while in *C. papillata* it is more clearly part of the ascomatal base. Furthermore, the ascomatal necks, the primary and secondary conidiophores, the cylindrical conidia and the chlamydospores of *C. papillata* are smaller than those reported for *C. caryae* (Johnson et al. 2005). *Ceratocystis smalleyi* has only one conidiophore type while *C. papillata* has both primary and secondary conidiophores. Further, *C. smalleyi* lacks the strong sweet banana aroma which is very evident in *C. papillata*. *Ceratocystis variospora* possess much larger ascomatal bases and longer ascomatal necks than *C. papillata*. *Ceratocystis populicola* is different from *C. papillata* in that both types of conidiophores are longer in *C. populicola* and the ascomatal necks are shorter for isolates in *C. populicola*.

*Ceratocystis colombiana* is closely related to *C. papillata* and morphologically similar to those species in the so-called "Latin-American" clade of *C. fimbriata s.l.* (Johnson et al. 2005). Unlike *C. papillata*, *C colombiana* does not have a single characteristic that distinguishes it from the other species in this group. In this case, reliance on DNA sequencing and phylogenetic placement is required to identify it with confidence.

*Ceratocystis colombiana* can be distinguished from *C. fimbriata s.s.* by the presence of the secondary conidiophores and secondary conidia that are absent in *C. fimbriata s.s.* The ascomatal bases of *C. colombiana* are smaller than those of *C. fimbriata s.s.* Other differences between these species are the shorter ascomatal necks, ostiolar hyphae and primary conidiophores of *C. colombiana* when compared with those of *C. fimbriata s.s.*

*Ceratocystis papillata* and *C. colombiana* have been isolated from a wide range of hosts including three cultivated tree crops as well as native trees in Colombia. Isolates from all three crop plants (coffee, cacao and citrus) were able to infect their hosts of origin, but also the other plants considered. There was thus no indication of host specificity of these two pathogens. Within *C. fimbriata s.l.*, some species are known to be highly host specific, infecting only a single host species, e.g. *C. platani* only infects plane trees (Johnson et al. 2005). However, there are pathogenic species in the *C. fimbriata s.l.* complex that are not host-specific, e.g. *C. albifundus* M.J. Wingf., De Beer & M.J. Morris which has been isolated from nine different host

genera (Roux et al. 2007). The relatively large number of species residing in the *C. fimbriata s.l.* species complex that are found in Latin American, suggests that this may be a centre of diversity for the group (Harrington 2000; Johnson et al. 2005). The natural hosts of *C. papillata* and *C. colombiana* could be native tree species and their ability to infect non-native crop plants might indicate that they are species that have wide host ranges in their native environment.

*Ceratocystis papillata* and *C. colombiana* are both virulent pathogens of coffee in Colombia, where this tree is one of the county's most important sources of income and employment. Recognising these fungi as distinct taxa could have significant implications for managing the diseases that they cause. Although they seem not to be host specific, selection of resistant cultivars will need to consider differences between these fungal species. Furthermore, Marin (2004) has shown that isolates of the two species tend to be limited predominately to a single orchard and to different areas of the country. Every effort should be made to reduce the chances of local spread of these species.

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