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Species delineation in the tree pathogen genus *Celoporthe* (Cryphonectriaceae) in southern Africa

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Abstract: The genus *Celoporthe* was first described when *C. dispersa* was discovered in South Africa associated with dieback and cankers on trees in the Myrtales. Four additional species were recently described from *Eucalyptus* and *Syzygium cumini* in China as well as *S. aromaticum* and *Eucalyptus* in Indonesia. Inoculation trials have shown that all *Celoporthe* species, including those that have not been found on *Eucalyptus* species in nature, are pathogenic to *Eucalyptus* and they are thus potentially threatening to commercial *Eucalyptus* forestry. New isolates, morphologically similar to *Celoporthe*, have been collected from *S. legatti* in South Africa and *S. guineense* in Zambia. Multigene phylogenetic analyses based on DNA sequences of the ITS region, TEF1α gene and two areas of the β-tubulin gene revealed additional cryptic species in *Celoporthe*. Phylogenetic data were supported by morphological differences. These resulted in the description of two previously unknown species of *Celoporthe*, namely *C. fontana* and *C. woodiana*, for two of these cryptic groups, while the third group represented *C. dispersa*. These species all can readily infect *Eucalyptus* as well as several species of *Syzygium*, the latter of which are native to Africa.

Key words: canker pathogens, *Heteropyxis canescens*, Myrtales, *Syzygium* species, *Tibouchina granulosa*

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Introduction

The genus *Celoporthe* (Cryphonectriaceae) was first described in 2006 and currently includes five species. These are *Celoporthe dispersa* Nakab., Gryzenh., Jol. Roux & M.J. Wingf. from South Africa and Zambia (Nakabonge et al. 2006a, Vermeulen et al. 2011), *C. eucalypti* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou, *C. guangdongensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou and *C. syzygii* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from China (Chen et al. 2011) and *C. indonesiensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from Indonesia (Chen et al. 2011). *Celoporthe* species are known only from hosts in the Myrtales (Nakabonge et al. 2006a, Chen et al. 2011, Vermeulen et al. 2011). The type species, *C. dispersa*, is known only from South Africa and Zambia where it occurs on native *Heteropyxis canescens* Oliv. (Heteropyxidaceae, Myrtales), *Syzygium cordatum* Oliv., *S. guineense* (CD.) and *S. legatti* Burtt Davy & Greenway (Myrtaceae, Myrtales) and non-native *Tibouchina granulosa* Cogn. : Britton (Melastomataceae, Mytales) (Nakabonge et al. 2006a, Vermeulen et al. 2011). *Celoporthe indonesiensis* infects *S. aromaticum* (L.) Merr & Perry in Indonesia (Myburg et al. 2003, Chen et al. 2011), while the Chinese species *C. eucalypti* and *C. guangdongensis* were collected from non-native *Eucalyptus* species and *C. syzygii* is known from non-native *S. cumini* (L.) Skeels (Chen et al. 2011).

Celoporthe dispersa is associated with cankers and branch dieback on *S. cordatum* and *T. granulosa* (Nakabonge et al. 2006a). Cankers on *Het. canescens*, from which *C. dispersa* was first collected, were severe with some trees dying. It was not shown however that *C. dispersa* was responsible for the death of these trees because pathogenicity tests could not be performed on *Het. canescens*. *Celoporthe dispersa* also poses a potential threat to *Eucalyptus* forestry in that inoculation trails have shown that the fungus is pathogenic to *Eucalyptus* species, although it has not been seen to infect these trees in nature (Nakabonge et al. 2006a).

Infections of *Eucalyptus* and *Syzygium* trees with *C. eucalypti*, *C. guangdongensis* and *C. syzygii* are associated with cracked bark and girdling stem cankers. It is not clear however whether these symptoms are associated with *Celoporthe* species only or whether symptoms are caused by *Chrysoporthe deuterocubensis* Gryzenh. & M.J. Wingf., a well known *Eucalyptus* pathogen (van der Merwe et al. 2010)

known to occur in China (Chen et al. 2010). These fungi co-occur on *Syzygium* and *Eucalyptus* in China (Chen et al. 2011), and it is possible that they combine to produce the symptoms observed in the field. Pathogenicity tests (Chen et al. 2011) have shown that Chinese *Celoporthe* species from *Syzygium* and *Eucalyptus* are as pathogenic to various *Eucalyptus* genotypes and *S. cumini* trees as *Chr. deuterocubensis* (Chen et al. 2010).

Nakabonge et al. (2006a) observed three distinct phylogenetic subclades (1–3) for *C. dispersa* isolates from South Africa (FIG. 1). These represented isolates from three different hosts and locations, namely *Het. canescens* (Lydenburg, Mpumalanga Province), *S. cordatum* (Tzaneen, Limpopo Province) and *T. granulosa* (Durban, KwaZulu-Natal Province). Isolates residing in these subclades were not described as distinct species because there were no obvious morphological differences observed between structures on the limited herbarium material available and no teleomorph structures were available for isolates from *Het. canescens* and *T. granulosa*. Vermeulen et al. (2011) observed two additional subclades, representing isolates from *S. cordatum* and *S. legatti* in South Africa (Soutpansberg, Limpopo Province) and from *S. guineense* in Zambia (Ikelenge, North Western Province) (FIG. 1). Due to the limited availability of specimens for the five subclades (1, 2) from Africa, comprehensive morphological comparisons between the collections were not possible at the time (Vermeulen et al. 2011).

The five distinct subclades in *Celoporthe* from Africa, observed by Nakabonge et al. (2006a) and Vermeulen et al. (2011), could represent five cryptic species similar to the species described from China and Indonesia (Chen et al. 2011). The aim of this study was to determine whether more than one species of *Celoporthe* is present in Africa based on newly available collections, multilocus DNA sequence data and morphological comparisons. Furthermore, we considered whether they differ in pathogenicity on a *Eucalyptus* clone and *S. cordatum* seedlings in the greenhouse.

MATERIALS AND METHODS

Fungal isolates.—Isolates of *C. dispersa* (Nakabonge et al. 2006a) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (TABLE I). Additional samples were obtained from surveys of native Myrtales, the only known hosts of *Celoporthe* species, conducted in the Soutpansberg (Limpopo Province) and Lydenburg (Mpumalanga Province) areas of South Africa. All suitable trees were visually inspected for signs of cankers, dead branch stubs and branches and examined for the presence of fungal fruiting bodies with a 10× magnification hand lens. Where present, pieces of bark bearing the fungal fruiting bodies resembling the Cryphonectriaceae were removed and placed in separate brown paper bags for transport to the laboratory. Isolations were made from pieces of bark bearing fruiting structures resembling those of *Celoporthe* species with techniques described by Gryzenhout et al. (2009). The cultures obtained were deposited in the CMW culture collection (TABLE I), and duplicates of selected isolates were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Herbarium specimens of fruiting structures on bark of selected fungi were also deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

DNA sequence comparisons.—DNA was extracted from mycelium grown on 2% malt extract agar (MEA). Mycelium was scraped from the surfaces of the MEA plates and freeze dried. Freeze-dried mycelium was ground to a fine powder with 2 mm diam metal beads in a Retsch cell disrupter (Retsch Gmbh, Germany) after which the protocol described by Möller et al. (1992) was followed for DNA extraction. DNA concentrations were determined with a NanoDrop 3.1.0 ND-1000 uv/Vis spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Four gene regions were amplified with the polymerase chain reaction (PCR). The β-tubulin 1 and β-tubulin 2 regions of the β-tubulin gene (BT) were amplified with primer pairs BT1a, BT1b and BT2a, BT2b respectively (Glass and Donaldson 1995). The internal transcribed spacer (ITS) regions (ITS1, ITS2) and the conserved 5.8S gene of the ribosomal RNA (rDNA) operon were amplified with the primer pair ITS1 and ITS4 (White et al. 1990). A portion of the elongation factor 1 α (TEF1α) gene was amplified with primers EF1-728 and EF986R (Carbone and Kohn 1999).

PCR reactions were performed in a total volume of 25 μL comprising 40 ng DNA template, 0.5 μM each primer, 0.2 mM each dNTP, 0.5 U Super-term polymerase Taq (Southern Cross Biotechnology, Cape Town, South Africa), 10× dilution buffer, 1 μL $MgCl_2$ (Southern Cross Biotechnology, Cape Town, South Africa) and sterile distilled water (18 μL). PCR were carried out on a thermal-cycler (Master cycle® Perkin Elmer Corp., Massachusetts) and included an initial denaturation step at 94 C for 3 min, followed by 40 amplification cycles consisting of 30 s at 94 C, 45 sec of annealing at 55 C for βT1, ITS and TEF1α, 65 C for BT 2, and 1 min at 72 C followed by 4 min at 72 C to ensure elongation of the fragments. PCR products were viewed with UV light on 1% agarose gels containing ethidium bromide to determine the presence or absence of bands. PCR products were cleaned using 0.06 g/mL Sephadex G-50 (SIGMA-ALDRICH, Amersham Biosciences Ltd., Sweden) following the manufacturer's instructions.

DNA fragments were sequenced with the same primer pairs used in the PCR amplification reactions. Sequencing reactions were performed in a volume of 10 μL consisting of 5× dilution buffer, 4 μL H_2O, DNA, 10× reaction mix and 2 pmol/μL primer. PCR sequencing products were cleaned as described above with Sephadex G-50 columns. The

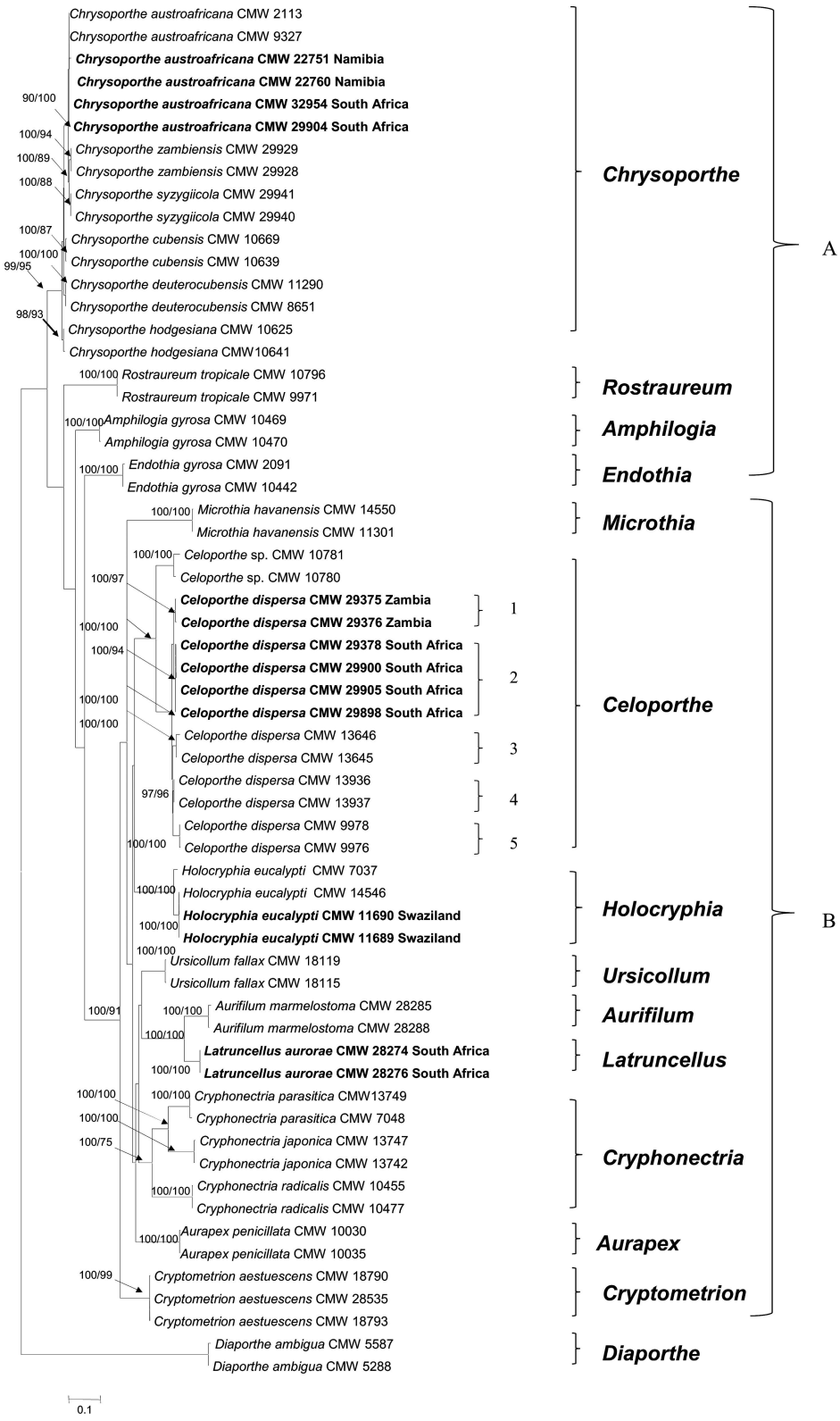

FIG. 1. Phylogram obtained from the combined datasets of the ITS and BT gene sequences (Vermeulen et al. 2010). The phylogram was obtained with maximum likelihood analyses using the TrN + I + G parameter model. Confidence levels > 70% of the tree branch nodes, determined by posterior probabilities (BPP) and ML, 1000 replicate bootstrap analysis (BS) are indicated on tree branches (BPP/BS). Isolates sequenced in this study are in boldface. Five clades of *Celoporthe dispersa* are marked 1–5. *Diaporthe ambigua* was defined as the outgroup taxon.

TABLE I. Isolates used in DNA sequence comparison, inoculations and pathogenicity trails

Identity	Isolate no. CMW [A]	CBS [B]	PREM [C]	Host	Country and province	Collectors	GenBank accession nos. [D]
Celoporthe dispersa	13645[E]	119119	58899	*H. canescens*	South Africa, Mpumalanga	G Nakabonge, J Roux and M Gryzenhout	DQ267134[1], DQ267140[2], DQ267146[2], JQ824062[3]
	32952			*H. canescens*	South Africa, Mpumalanga	M Vermeulen, M Gryzenhout	JQ8240776[1], JQ824084[2], JQ824069[3]
	32951			*H. canescens*	South Africa, Mpumalanga	M Vermeulen, M Gryzenhout	JQ824076[1], JQ824083[2], JQ824068[3]
	9978[E]	118781	58896	*S. cordatum*	South Africa, Limpopo	M Gryzenhout	AY214316[1], DQ267135[2], DQ267141[2], HQ730841[3]
	9976[E]	118782	58897	*S. cordatum*	South Africa, Limpopo	M Gryzenhout	DQ267130[1], DQ267136[2], DQ267142[2], HQ730840[3]
	9977[E]			*S. cordatum*	South Africa, Limpopo	M Gryzenhout	JQ824075[1], JQ824080[2], JQ824061[3]
	29378			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726942[1], GU726954[2], JQ824063[3]
	29898			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726943[1], GU726955[2], JQ824064[3]
	29900			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726944[1], GU726956[2], JQ824065[3]
	29901			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	JQ824078[1], JQ824081[2], JQ824067[3]
	29903			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	JQ824079[1], JQ824082[2], JQ824070[3]
	29905			*S. cordatum*	South Africa, Limpopo	M Vermeulen, J Roux	GU726945[1], GU726957[2], JQ824066[3]

TABLE I. Continued

Identity	Isolate no. CMW [A]	CBS [B]	PREM [C]	Host	Country and province	Collectors	GenBank accession nos. [D]
C. eucalypti	26900	127191	60466	*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730836[1], HQ730816[2], HQ730826[2], HQ730849[3]
	26908	127190	60467	*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730837[1], HQ730817[2], HQ730827[2], HQ730850[3]
	26911	127192		*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730838[1], HQ730818[2], HQ730828[2], HQ730851[3]
	26913			*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730839[1], HQ730819[2], HQ730829[2], HQ730852[3]
C. fontana	29375			*S. guineense*	Zambia, North Western	M Vermeulen, J Roux	GU726940[1], GU726952[2], JQ824073[3]
	29376			*S. guineense*	Zambia, North Western	M Vermeulen, J Roux	GU726941[1], GU726953[2], JQ824074[3]
C. guangdongensis	12750	128341	60468	*Eucalyptus* sp.	China, GuangDong	TI Burgess	HQ730830[1], HQ730810[2], HQ730820[2], HQ730843[3]
C. indonesiensis	10781	115844	60469	*S. aromaticum*	Indonesia, North Sumatra	MJ Wingfield	AY084009[1], AY084021[2], AY084033[2], HQ730842[3]
	10779			*S. aromaticum*	Indonesia, Somosir	MJ Wingfield	AY084007[1], AY084019[2], AY084031[2]
	10780			*S. aromaticum*	Indonesia, Somosir	MJ Wingfield	AY084008[1], AY084020[2], AY084032[2]

TABLE I. Continued

Identity	Isolate no. CMW [A]	CBS [B]	PREM [C]	Host	Country and province	Collectors	GenBank accession nos. [D]
C. syzygii	34023	127218	60462	*S. cumini*	China GuangDong	SF Chen	HQ730831[1], HQ730811[2], HQ730821[2], HQ730844[3]
	34024		60463	*S. cumini*	China GuangDong	SF Chen	HQ730832[1], HQ730812[2], HQ730822[2], HQ730845[3]
	24912	127188	60464	*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730833[1], HQ730813[2], HQ730823[2], HQ730846[3]
	24914	127189	60465	*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730834[1], HQ730814[2], HQ730824[2], HQ730847[3]
	24917			*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730835[1], HQ730815[2], HQ730825[2], HQ730848[3]
C. woodiana	13936[E]	118785		*T. granulosa*	South Africa, Kwazulu-Natal	M Gryzenhout	DQ267131[1], DQ267137[2], DQ267143[2], JQ824071[3]
	13937[E]			*T. granulosa*	South Africa, Kwazulu-Natal	M Gryzenhout	DQ267132[1], DQ267138[2], DQ267144[2], JQ824072[3]

[A] Culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[B] CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

[C] PREM, Agricultural Research Council, Pretoria, South Africa.

[D] GeneBank accession numbers for sequence data of the ITS[1] (primers ITS1/2), BT[2] 1 and 2 (primers BT1a/1b and BT21/2b) and Translation elongation factor 1-alpha[3] positions (primers EF1-728 and EF986R).

[E] ITS and BT of these isolates were sequenced and re-submitted to GeneBank.

products were sequenced in both directions with the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, California) on an ABI Prism™ 3100 DNA sequencer (Applied Biosystems).

The presence of a mononucleotide repeat (T) upstream of the ITS primer binding site in several isolates (TABLE I) caused slipped-strand miss-pairing (Fazekas et al. 2010). This resulted in the formation of stutter products, after which the quality of sequence data is greatly reduced. To obtain these sequences, PCR products were cloned into pGEM®-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* JM109 high efficiency competent cells (Promega) following the supplier's instructions. Plasmid DNA was prepared with the alkaline lyses method (Sambrook and Russel 2006) and sequenced with primer pair SP6 and T7 (Promega) as described above. A second mononucleotide (A) repeat was present upstream of the Mononucleotide (T) repeat in the ITS, and there were also mononucleotide repeats in the BT (C) and TEF1α (T) regions. Sequence data for these regions could be obtained from both primer binding sites up to the repeat, and forward and reverse sequences were repeated to validate the sequence data.

Gene sequences were viewed and edited with CLC Main Workbench, CLC BIO 5.5 (CLC bio A/S, Science Park Aarhus, Finlandsgade 10–12, 8200 Aarhus N, Denmark). The National Centre for Biotechnology Information (NCBI) database was accessed and preliminary identifications were obtained for sequences with the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned with published sequences of closely related fungal species (TABLE I) with the Web interface (http://mafft.cbrc.jp/alignment/server/) of the alignment program MAFFT 5.8 (Katoh et al. 2002). The alignments were deposited at TreeBASE (www.treebase.org) (submission 12391).

Phylogenetic analyses were performed with the software package PAUP* 4.01b10 (Swofford 2000). Phylogenetic analyses were done for each gene region separately with maximum parsimony (heuristic search with 100 random sequence additions). A 1000 bootstrap replication was performed, to determine the support of branches for the most parsimonious tree for datasets representing each of the gene regions (Felsenstein 1985). A 1000-replicate partition homogeneity test (PHT) was performed to determine the null hypotheses that the BT, ITS and TEF1α gene datasets were homologous and could be combined for further analyses (Farris et al. 1994). PHT was done after the exclusion of uninformative sites, with a heuristic search with 100 random sequence additions, tree bisection-reconnection (TBR) branch swapping and MAXTREES set to 5000 to allow completion of analysis.

A second phylogenetic analysis was done based on maximum likelihood (ML) for each gene region separately and for the combined dataset. The correct models for the datasets were identified using jModeltest 0.0.1 (Posada 2008). The TrNef + I model (Posada 2008) was shown to be appropriate for the ITS, the TIM3 + G model (Tavaré 1986) for BT, the HKY + I model (Hasegawa et al. 1985) for TEF1α and the TIM2 + G model (Tavaré 1986) for the combined dataset. Maximum likelihood analyses were performed with PhyML 3 (Guindon and Gascuel 2003). A 1000-replicate bootstrap analysis was done to assess the confidence of the branch nodes in the phylogenetic trees.

Morphology.—To study the morphology of isolates for which herbarium specimens were not available, representative isolates for each phylogenetic clade (TABLE I) were inoculated into sterile *Eucalyptus* clone ZG14 and *S. cordatum* stem sections and incubated on water agar to induce sporulation (Gryzenhout et al. 2009). *Eucalyptus* clone ZG 14 and *S. cordatum* seedlings were cut into 6 cm sections and autoclaved. The bark was removed from the stem sections with a 5 mm cork borer to expose the cambium layer and mycelium plugs from 7 d old cultures were placed, mycelium facing downward, into the wounds. The stem sections were placed onto water agar and incubated at 25 C for 6 wk. In addition, mycelium-bearing plugs were taken from 7 d old cultures, placed on oatmeal agar and incubated at 25 C for 6 wk in an attempt to induce sporulation. Furthermore, *S. cordatum* seedlings were inoculated as described below in the pathogenicity section and kept at 25 C for 6 wk to induce sporulation.

Fruiting structures that developed on *Eucalyptus* clone ZG14 and *S. cordatum* stem sections, oatmeal agar and *S. cordatum* seedlings, as well as those on original bark material collected from the field, were cut from the bark under a dissection microscope. Thin sections of resultant fruiting structures were made by hand and crushed on microscope slides in 3% potassium hydroxide (KOH) to observe conidia, conidiophores and conidiogenous cells (Gryzenhout et al. 2009). Twenty measurements of each of the above mentioned structures were taken and are presented as (min–)(average-SD)–(average + SD)(–max) mm. Fifty measurements of asci, ascospores, conidia, conidiophores and conidiogenous cells were taken for fruiting structures on bark from natural infections. Digital images were captured with a HRc Axiocam digital camera and measurements were computed using Axiovision 3.1 software (Carl Zeiss Ltd., Germany). Characteristics of fruiting bodies were compared to characteristics published for *Celoporthe* species (Nakabonge et al. 2006a, Gryzenhout et al. 2009, Chen et al. 2011).

Characteristics of growth in culture for representative isolates (TABLE I) in the subclades were assessed. Disks were taken from the edges of actively growing cultures on MEA and transferred to the middles of 90 mm Petri dishes containing MEA. Five plates per isolate were prepared, and these were incubated in the dark 15–35 C. Two diameter measurements perpendicular to each other were taken for each colony until the fastest growing culture had covered the plate. Color notations of Rayner (1970) were used to describe cultures.

Pathogenicity tests.—Pathogenicity tests were conducted with isolates representative of the subclades identified with DNA sequence data (TABLE I). Ten 2 y old plants of *Eucalyptus* clone ZG14 that had been shown to be highly susceptible to both *Chr. austroafricana* and *C. dispersa* (van Heerden and Wingfield 2001, Nakabonge et al. 2006a) and 10 *S. cordatum* trees were inoculated with each isolate. Trees

were kept at 25 C in a greenhouse under natural day/night conditions for 2 wk to acclimatize, after which they were inoculated. For inoculations, a bark disk was removed from each tree with a 5 mm cork-borer, and a plug of mycelium of the test fungus taken from the actively growing margin of a culture was placed into the wound, with the mycelium facing the cambium. For controls, sterile MEA plugs were placed into the wounds. Wounds were sealed with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum plugs.

Six wk after inoculation the bark associated with the inoculation sites was removed and the lengths of lesions on the cambium were measured. Pieces of necrotic tissue were transferred to MEA to re-isolate the inoculated fungi. The pathogenicity trial was repeated once under the same conditions. Variation in lesion lengths was assessed in SAS® 8.2 with the general linear model command (PROC GLM) (SAS Institute 1999).

RESULTS

Fungal isolates.—Ten isolates previously identified as *C. dispersa* were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (TABLE I) and two from herbarium specimens from Zambia (Vermeulen et al. 2011). Two additional isolates with newly collected herbarium material were obtained during field collections from *S. legatti* in Soutpansberg (South Africa) and two from *Het. canescens* in Lydenburg (South Africa) from the same area as those from Nakabonge et al. (2006a) and Vermeulen et al. (2011). Infections on *S. legatti* in Soutpansberg and on *Het. canescens* in Lydenburg were relatively inconspicuous with infections found on branch stubs and branch cankers. Despite continued and widespread surveys, also in other areas of southern Africa, these were the only additional specimens that could be obtained.

DNA sequence comparisons.—Sequence datasets consisted of 27–31 taxa (TABLE I) and the aligned DNA sequence dataset varied from 268 bp for the BT dataset to 1577 bp for the combined ITS, BT and TEF1α dataset (TABLE II). Results of the PHT showed that datasets for the three gene regions were homologous ($P = 0.072$) and could be combined (Cummings et al. 1995). This also was supported by the trees for each gene region that essentially had the same topology and support for the various clades. Based on the ITS, βT and TEF1α datasets individually (FIG. 2a–c), as well as the combined dataset (FIG. 2d), isolates from Africa grouped in three distinct clades.

The African clades grouped separately from the four new species described from Asia (Chen et al. 2011). Isolates from *S. legatti* (Limpopo Province, South Africa) and *Het. canescens* (Mpumalanga Province, South Africa) grouped with the type specimen of *C. dispersa* (MP and ML, 1000 replicate bootstrap analysis indicated as [MP/ML], 99/100). Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa) (99/100) and *S. guineense* (North Western Province, Zambia) (98/100) formed two additional subclades. Results of the parsimony analyses correlated with those for the maximum likelihood analyses. The three subclades representing the southern African isolates were observed for all gene regions separately as well as in the combined datasets (FIG. 2a–d).

The different phylogenetic subclades observed were associated with uniquely fixed DNA nucleotides (TABLE III). Several differences in the number of mononucleotide repeats were observed in non-protein coding regions for sequences of the ITS, BT and TEF1α regions. These were excluded from the aligned datasets because the true repeat number could not be confirmed.

Morphology.—Most isolates representing the three African subclades (FIG. 2d) could be induced to sporulate when inoculated into sterile *Eucalyptus* clone ZG14 and *S. cordatum* stem sections incubated on water agar. However, only anamorph structures were found. Conidiomata resulting from the three techniques to induce sporulation differed in shape

TABLE II. Statistics resulting from maximum parsimony analyses

Statistic	ITS	BT	TEF1α	Combined gene regions
Number of taxa	31	31	27	27
Aligned characters	497	812	268	1577
Constant characters	425	696	248	
Parsimony-uninformative characters	14	7	3	
Parsimony-informative characters	58	109	17	
Tree length	94	140	22	113
Consistency index (CI)	0.989	0.921	0.955	0.920
Retention Index (RI)	0.998	0.979	0.992	0.988
Rescaled consistency index (RC)		0.902	0.947	0.910

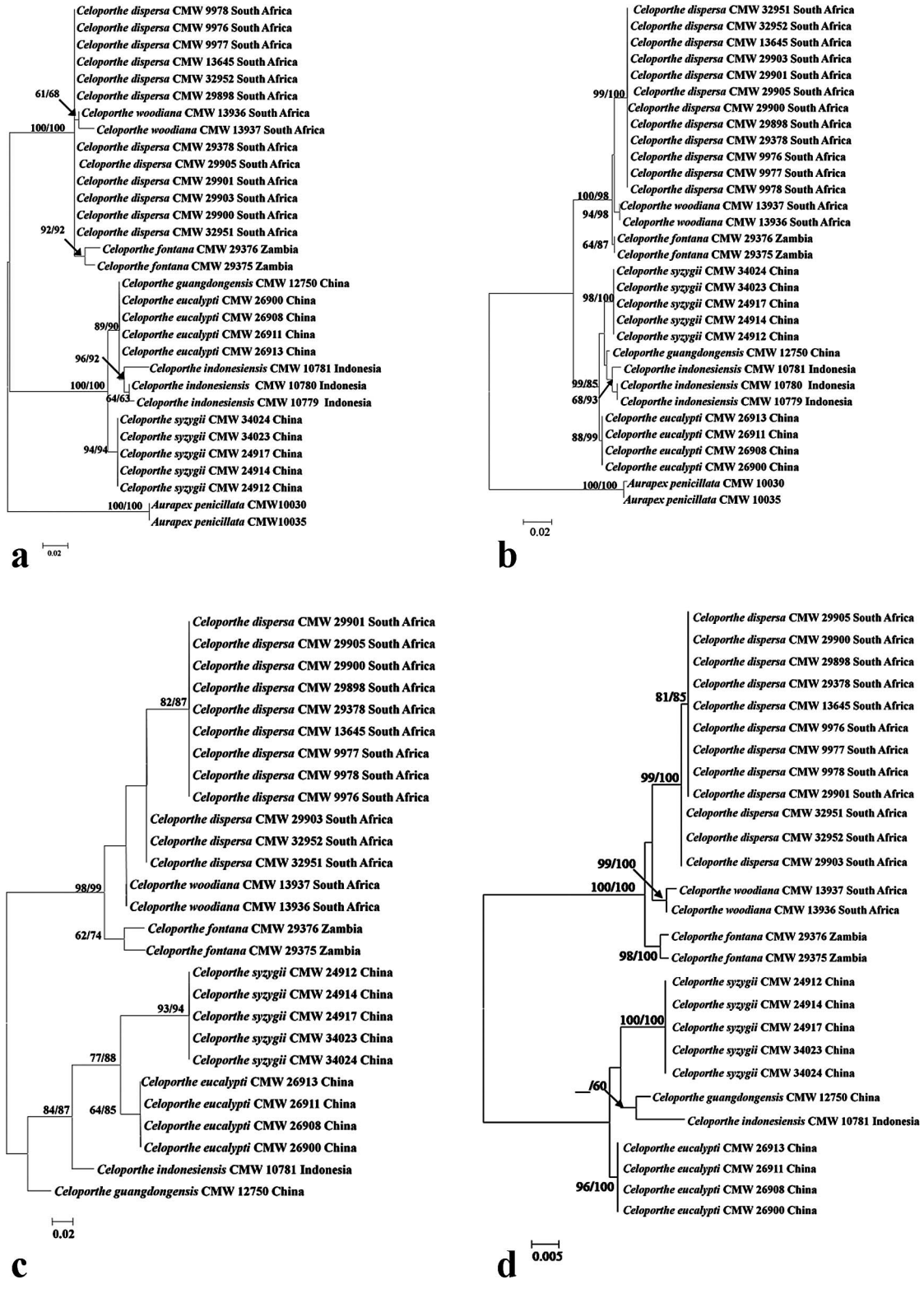

FIG. 2. Phylograms obtained from maximum likelihood analyses. a. Phylogram for ITS gene sequences, obtained with the TrNef + I parameter model. b. Phylogram for BT gene sequences, obtained with the TIM3 + G parameter model. c. Phylogram for TEF1α gene sequences, obtained with the HKY + I parameter model. d. Phylogram for combined ITS, BT and TEF1α gene sequences, obtained with the HKY + I parameter model. For all trees confidence levels > 60% (1000 replicate bootstrap analysis) of the tree branch nodes determined by MP and ML are indicated on tree branches (MP/ML). a–b. *Aurapex penicillata* defined as the outgroup taxon. c–d. Trees were midpoint rooted.

TABLE III. Unique fixed DNA nucleotides in ITS, BT and TEF1α sequences for African *Celoporthe* clade

Species	Isolate number	ITS				BT												TEF1α			
		111	176	177	412	133	142	177	179	180	182	189	194	243	632	633	645	31	164	207	217
C. dispersa	9978	−	A	A	T	C	T	A	C	C	C	C	T	C	T	T	C	A	A	G	−
C. dispersa	9976	−	A	A	T	C	T	A	C	C	C	C	T	C	T	T	C	A	A	G	−
C. woodiana	13936	−	A	A	C	T	C	C	T	T	T	T	A	C	T	C	A	A	A	A	−
C. woodiana	13937	−	A	A	C	T	C	C	T	T	T	T	A	C	T	C	A	A	A	A	−
C. fontana	29375	G	T	G	T	C	C	C	T	T	T	C	A	T	C	C	C	G	T	A	T
C. fontana	29376	G	T	G	T	C	C	C	T	T	T	C	A	T	C	C	C	G	T	A	T

and position, relative to bark specimens from natural infections, consistent with observations of Chen et al. (2011). However, the conidia, conidiogenous cells and paraphyses resulting from inoculations were similar to those for natural infections.

There were bark specimens for *Het. canescens* in Lydenburg, *S. legatti* in Soutpansberg and *S. guineense* in Zambia. Fruiting structures on bark resembled those of *C. dispersa* (Nakabonge et al. 2006a, Gryzenhout et al. 2009). Only teleomorph structures were present on bark specimens from *Het. canescens* in Lydenburg, and only anamorph structures were present on bark specimens from *S. legatti* in Soutpansberg and *S. guineense* in Zambia. Teleomorph structures on *Het. canescens* from Lydenburg and anamorph structures from *S. legatti* in Soutpansberg were similar to those previously described for *C. dispersa* (TABLE II). However, anamorph structures from *S. guineense* (Zambia) differed from specimens on *Het. canescens* from Lydenburg and those described for *C. dispersa* (Nakabonge et al. 2006a) because the conidiomata were multilocular, whereas those of *C. dispersa* and specimens on *Het. canescens* from Lydenburg and Soutpansberg typically were unilocular. The conidiomatal and ascostromatal morphology of the *T. granulosa* (KwaZulu-Natal Province, South Africa) group of isolates could not be assessed as specimens representing naturally infected tissue could not be obtained.

Structures arising from inoculated material revealed morphological differences corresponding to the three subclades from Africa defined based on DNA sequence data. These three subclades (FIG. 2d) could be differentiated based on conidial size, although in general there was an overlap between characteristics of conidia, conidiogenous cells and paraphyses. Isolates representing the *C. dispersa* s.s. subclade had longer (max 5.5 µm) and wider conidia (max 2.5 µm) than those representing the subclade from *T. granulosa* (KwaZulu-Natal Province, South Africa) (max 4.5 µm × 2 µm) and wider conidia than isolates from *S. guineense* in Zambia (max 6 µm × 2 µm). Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa) had conidia that were oblong to cylindrical and occasionally allantoid (FIG. 3i), while those from *S. guineense* in Zambia had conidia that were oblong to cylindrical and occasionally ovoid (FIG. 3f). Additional differences between isolates representing the subclades were those relating to growth in cultures. The optimal temperature for growth of isolates from *S. guineense* (Zambia) was 30 C while those from South Africa grew optimally at 25 C. Colony color and texture of the isolates were similar however.

TAXONOMY

Based on phylogenetic analyses of DNA sequence data for three gene regions, isolates of *Celoporthe* from Africa resided in three different sub-clades, separate from recently described species from China and Indonesia. These three sub-clades can be identified based on uniquely fixed nucleotides (TABLE III), a combination of morphological characteristics from naturally infected as well as inoculated tissue and geographic separation. Isolates from Soutpansberg and Lydenburg grouped with those of the type specimens for *C. dispersa* from *S. cordatum* (Limpopo Province, South Africa). Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa), however, grouped separately from other isolates and had conidia that are oblong to cylindrical, occasionally allantoid. Isolates from *S. guineense* in Zambia grouped separately based on all three gene regions, are from an area geographically separated from the other groups and they were also morphologically distinct. These morphological differences included multilocular as opposed to unilocular conidiomata, and conidia that are oblong to cylindrical and occasionally ovoid. The African isolates considered in this study, therefore, represent three distinct species including *C. dispersa* and two previously unrecognized species described as follows:

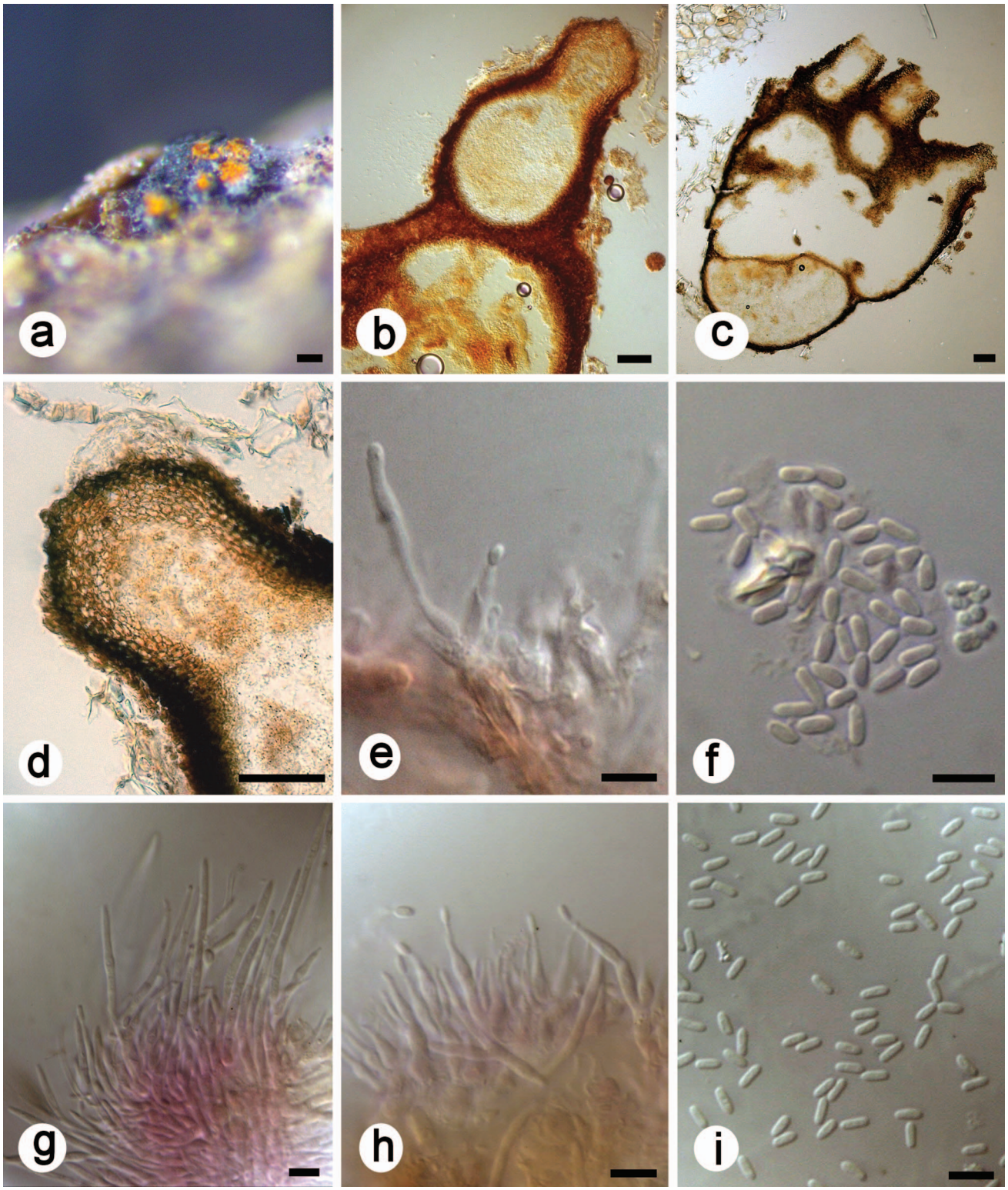

FIG. 3. Fruiting structure of *Celoporthe fontana* and *C. woodiana*. a–f. Fruiting structures of *C. fontana* on *Syzygium guineense* trees infected in the field. a. Conidioma on the bark. b–c. Longitudinal sections through conidiomata. d. Stromatic tissue of conidioma. e. Paraphyses and conidiogenous cells. f. Conidia. g–i. Fruiting structures of *C. woodiana* on greenhouse inoculated stem tissue of *S. cordatum*. g. Paraphyses. h. Conidiogenous cells. i. Conidia. Bars: a = 100 µm, b–d = 50 µm, e–i = 5 µm.

Celoporthe fontana M. Verm., Gryzenh. & Jol. Roux., sp. nov. FIG. 3a–f

MycoBank MB563045

Etymology: fontana (Latin) ''of the spring'' referring to the origin of the Zambezi River, which is close to the type locality of this species.

Teleomorph: Not known.

Conidiomata: Herbarium specimen: Globose, conical to pulvinate, semi-immersed, fuscous to black, conidiomatal bases above the bark surface 290–380 µm high, conidiomatal 280–590 µm diam. Conidiomatal locules with even to convoluted inner surfaces, multilocular. Stromatic tissue pseudoparenchymatous, cinnamon to brown. Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, (5.0–)7.5–14.0(–22.5) × (1.0–)1.5(–2.0) µm. Paraphyses occurring between conidiophores, up to 77 µm long. Conidia (3.0–)3.5(–4.0) × 1.0–1.5 µm, hyaline, non-septate, oblong to cylindrical, occasionally ovoid, with rounded apexes, exuded as tendrils or droplets.

On inoculated material: Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, on *S. cordatum* stem sections (7.5–)9.0–29.0(–26.0) × 2(–3) µm, on *Eucalyptus* ZG14 stem sections (6.0–)7.5–16.0(–23.0) × (1.0–)1.5–2.0(–2.5) µm, on oats agar (10.5–)11.0–22.5(–21.5) × 2(–4) µm and on *S. cordatum* trees (7.0–)9.5–16.5(–19.0) × (1.0–)1.5(–2.0) µm. Paraphyses occurring between conidiophores, up to 88 µm long on *S. cordatum* trees. Conidia on *S. cordatum* stem sections (3.5–)4.5–5.0(–5.5) × (1.0–)1.5–1.5(–2.0) µm, on *Eucalyptus* ZG14 stem sections (4.0–)4.5–5.5(–6.0) × 1.5(–2.0) µm, on oats agar (3.5–)4.5(–5.0) × 1.5 µm and on *S. cordatum* trees (3.0–)3.5(–4.0) × (1.0–)(–1.5) µm, hyaline, non-septate, oblong to cylindrical, occasionally ovoid, with rounded apexes, exuded as tendrils or droplets.

Fixed base pair differences: Celoporthe fontana differs from other species in the African clade of *Celoporthe* (*C. dispersa* and *C. woodiana*) by uniquely fixed DNA nucleotides in three nuclear loci: β-tubulin 1 and 2 positions 243 (T) and 632 (C); internal transcribed spacer rDNA (ITS1, 5.8S, ITS2) positions 111 (G), 176 (T) and 177 (G); translation elongation factor 1-alpha positions 31 (G), 164 (T) and 217 (T).

Culture characteristics: Cultures on MEA white with gray patches, fluffy with uneven margins, changing from umber to hazel to chestnut, fast-growing, covering a 90 mm diam plate in 4–5 d at the optimum temperature of 30 C.

Specimens examined: ZAMBIA, NORTH WESTERN PROVINCE: Kaleni Hills, Ikelenge. Isolated from bark of *S. guineense*, 2006, M. Vermeulen and J. Roux. (HOLOTYPE, PREM60731 ex-type culture CMW29376/CBS132008, PARATYPE, PREM60732, (isolate CMW29375 artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), living culture CMW29375/CBS132007).

Celoporthe woodiana M. Verm., Gryzenh. & Jol. Roux. sp. nov. FIG. 3g–i

MycoBank MB563046

Etymology: Named after Dr Medley Wood, one of the first mycologists in South Africa who worked in the Durban Botanic Gardens. This location represents the only locality where this species has been found.

Teleomorph: Not known

Conidiomata. On herbarium specimens: not available. *On inoculated material:* Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, on *S. cordatum* stem sections (6.0–)8.5–16.5(–21.5) × (1.0–)1.5–2.0(–2.5) µm, on *Eucalyptus* ZG14 stem sections (5.0–)6.0–13.0(–17.0) × (1.0–)1.5–2.0(–2.5) µm and on oats agar (8.0–)8.5–17.5(–25.0) × (1.5–)2.0(–3.0) µm. Paraphyses occurring between conidiophores, up to 55 µm long on *S. cordatum* trees. Conidia on *S. cordatum* stem sections (3.0–)3.5–4.0(–4.5) × (1.0–)1.5(–2.0) µm, on *Eucalyptus* ZG14 stem sections 4.0–4.5 × (1.0–1.5) µm and on oats agar (3.5–)–4.0(–4.5) × 1.0–1.5 µm, hyaline, non-septate, oblong to cylindrical, occasionally allantoid, with rounded apex, exuded as luteous tendrils or droplets.

Fixed base pair differences: Celoporthe woodiana differs from other species in the African clade of *Celoporthe* (*C. dispersa* and *C. fontana*) by uniquely fixed DNA nucleotides in two nuclear loci: β-tubulin 1 and 2 positions 133 (T) 189 (T) and 645 (A); internal transcribed spacer rDNA (ITS1, 5.8S, ITS2) position 412 (C).

Culture characters: Cultures on MEA white with gray patches, fluffy with uneven margins, changing from umber, hazel to chestnut, fast-growing, covering 90 mm diam plates in 4–5 d at the optimum temperature of 25 C.

Specimens examined: SOUTH AFRICA, KWAZULU-NATAL: Durban Botanical garden. Isolated from bark of *T. granulosa*, 2006, M. Gryzenhout. (HOLOTYPE, PREM60734 (isolate CMW13936 artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), ex-type culture CMW13936/CBS118785, PARATYPE, PREM60733 (isolate CMW13937 artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), living culture CMW13937/CBS119118).

DICHOTOMOUS KEY TO *CELOPORTHE* SPECIES (based on anamorph structures only)

1a. Optimal growth at 25 C, conidiomata unilocular* . . . 2
1b. Optimal growth at 30 C, conidiomata multilocular . . . 3

2a. Conidial length shorter than 4.5 μm . . . *C. woodiana*
2b. Conidial length can be longer than 4.5 μm . . . . . *C. dispersa*
3a. Conidia, cylindrical, occasionally oblong . . . . . *C. indonesiensis*
3b. Conidia oblong to cylindrical 4
4a. Conidia occasionally ovoid . . . . . *C. fontana*
4b. Conidia occasionally allantoid 5
5a. Conidia shorter than 4 μm . . . . . *C. syzygii*
5b. Conidia longer than 4 μm 6
6a. Paraphyses shorter than 70 μm . . . . . *C. eucalypti*
6b. Paraphyses longer than 90 μm . . . *C. guangdongensis*

*Not known for *C. woodiana*.

Pathogenicity tests.—Six weeks after inoculation, lesions were visible on all trees other than those inoculated with sterile MEA plugs. Statistical analyses showed significant differences in lesion lengths on trees inoculated with the test fungi and the controls for both *Eucalyptus* clone ZG14 and *S. cordatum* ($P < 0.05$). Data for the two trails could not be combined, in that there were statistical differences between repeats ($P > 0.05$) (FIG. 4).

No statistical differences were observed between the lengths of lesions formed on the *Eucalyptus* clone ZG14 by *C. dispersa* (av. = 29.8 mm), *C. fontana* (av. = 30.1 mm) and *C. woodiana* (av. = 30.5) ($P > 0.05$) in either of the replications of the trial. Statistical differences, however, were observed for lesion lengths on *S. cordatum* ($P < 0.05$) and the results for the first and second repeat differed (FIG. 4). In both experiments the lesions formed on *Eucalyptus* clone ZG14 were longer than those on *S. cordatum*. Fruiting structures formed on inoculated stems of both tree species represented the inoculated fungi, thus fulfilling Koch's postulates.

DISCUSSION

In this study two previously undescribed species of *Celoporthe* from native *S. guineense* and non-native *T. granulosa* trees, were identified from Africa. *Celoporthe woodiana* originally was isolated from *T. granulosa* (KwaZulu-Natal Province, South Africa) but was treated as *C. dispersa* due to a lack of morphological characteristics to distinguish it (Nakabonge et al.

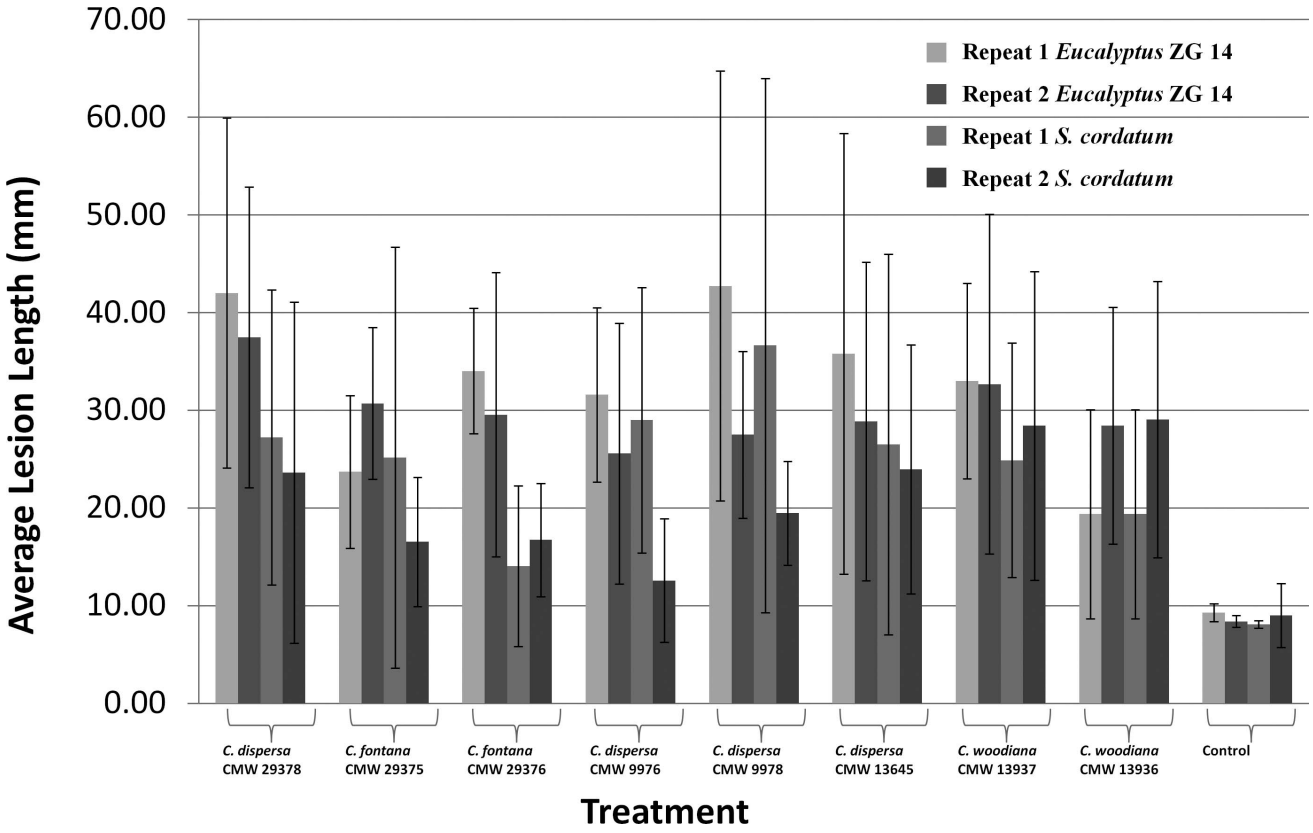

FIG. 4. Column chart indicating the average lesion length (in millimeters) resulting from inoculation trials for repeat one and two on a *Eucalyptus* ZG 14 clone and *Syzygium cordatum* seedlings under glasshouse conditions. Error bars represent standard deviation for each treatment.

2006a). *Celoporthe fontana* was isolated recently from *S. cordatum* in Zambia, but this species also was not described at the time of collection due to a lack of available herbarium specimens to allow for morphological comparisons (Vermeulen et al. 2011). A combination of morphology from naturally infected material and inoculated specimens as well as multigene phylogenies generated in this study made it possible to describe these *Celoporthe* species. The known distribution of *C. dispersa* was expanded and it currently includes isolates from the Limpopo and Mpumalanga Provinces in South Africa, on *S. cordatum* and *S. legatti* (Nakabonge et al. 2006a, Vermeulen et al. 2011).

Morphological characteristics provide little opportunity to distinguish among species of *Celoporthe*. This is because few specimens are available for examination and often only one of the morphological states is present (Nakabonge et al. 2006a, Chen et al. 2011, Vermeulen et al. 2011). Morphological studies of the Cryphonectriaceae are best when they are based on naturally infected tissues (Gryzenhout et al. 2009) because fruiting structures produced in culture or where host tissue is artificially inoculated usually exhibit variations in structure, size and shape (Hodges et al. 1986; Myburg et al. 2002, 2003; Chen et al. 2011). Thus, the shape and position of conidiomata of *Celoporthe* species produced on inoculated stems in this study also differed from those collected in the field. It therefore is important to use measurements of these structures with caution and where possible to cross compare them to those from naturally infected tissue (Kobayashi 1970).

This study relied strongly on the phylogenetic species concept (PSC) (Taylor et al. 2000) to recognize the new species *C. fontana* and *C. woodiana*. This is similar to the approach recently taken to recognize *Chr. deuterocubensis* as distinct from the well known *Eucalyptus* pathogen *Chr. cubensis* (van der Merwe et al. 2010). Chen et al. (2011) similarly described *C. eucalypti*, *C. guangdongensis*, *C. indonesiensis* and *C. syzygii* based on single base pair differences between species and supporting but overlapping morphological differences.

Results of this study add substance to the view that *Celoporthe* species have a widespread, yet possibly structured, occurrence in Africa and Asia. Isolates from Asia and Africa group separately, indicating possible geographical boundaries. In Asia, species currently are known only from China (three species) and Indonesia (one species). Given that there have been few studies of this group in that area (Chen et al. 2011) the diversity of these fungi in Asia most likely has been only partially sampled and nothing is known of their origin. Furthermore, none of the recorded hosts of the *Celoporthe* species found in Asia were native to the areas where they were collected. This is different from the situation in Africa, where surveys for Cryphonectriaceae have been undertaken widely in South Africa and in other African countries such as Kenya, Mozambique and Namibia (Nakabonge et al. 2006a, b; Vermeulen et al. 2011). Only three *Celoporthe* species were found in these surveys, of which two occur on native trees. *Celoporthe fontana* was found only in the North West Province of Zambia and *C. woodiana* only in the KwaZulu-Natal Province of South Africa. It seems likely that these species are native to southern Africa.

Pathogenicity tests in this study showed that *C. dispersa*, *C. fontana* and *C. woodiana* are pathogenic to *Eucalyptus* clone ZG 14 and *S. cordatum* seedlings under greenhouse conditions. The variation observed in lesion lengths for *C. dispersa*, *C. fontana* and *C. woodiana* on *S. cordatum* between the two inoculation trials could be due to differences in host resistance in that the plants were propagated from seed and individuals, therefore, would differ in susceptibility. However, the results provide clear evidence of pathogenicity in these fungi and they could emerge as important pathogens of *Eucalyptus* in the future, as has been shown with *Chr. austroafricana* (Wingfield 2003, Gryzenhout et al. 2009).

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