Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia

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Abstract

Among fungi in the Cryphonectriaceae, *Cryphonectria parasitica*, the causal agent of chestnut blight, and *Chrysoporthe* spp., inducing stem cankers and death of plantation-grown *Eucalyptus* spp., constitute some of the most important tree pathogens. During a survey to determine diseases and related pathogens associated with native and non-native Myrtales in Zambia, two fungi resembling stem canker pathogens in the genus *Chrysoporthe* were recovered from *Syzygium guineense* and *Eucalyptus grandis*. We undertook this study to characterise the fungi, using DNA sequence comparisons and morphological features. Inoculation tests were also conducted in a greenhouse to assess their pathogenicity on *Eucalyptus* spp. Results showed distinct phenotypic differences between isolates collected from Zambia and existing species of *Chrysoporthe*, and phylogenetic analyses demonstrated that the Zambian isolates represent previously undescribed species of *Chrysoporthe*. These isolates, which have been named *Chrysoporthe zambiensis* sp. nov. and *Chrysoporthe syzygiicola* sp. nov., are only known from their anamorphic states. Both species were found to be pathogenic to *Eucalyptus*. The description of *Chr. zambiensis* and *Chr. syzygiicola* with teleomorph names, led to the new combination *Chrysoporthe hodgesiana* for the only anamorphic species in *Chrysoporthe*, *Chrysoporthella hodgesiana*, to ensure more stable and less confusing taxonomy for *Chrysoporthe*.

Introduction

The Cryphonectriaceae is a recently established fungal family in the Diaporthales that includes several genera accommodating important tree pathogens (Gryzenhout *et al*. 2006a, 2009). For example, *Cryphonectria parasitica*, the causal organism of chestnut blight, has devastated the American chestnut (*Castanea dentata*) in North America and European chestnut (*Castanea sativa*) in Europe (Anagnostakis 1987; Heiniger and Rigling 1994). Similarly, *Chrysoporthe cubensis* and *Chr. austroafricana* have been reported as economically important pathogens of *Eucalyptus* spp. and other Myrtales in many tropical and sub-tropical countries (Gibson 1981; Hodges *et al*. 1985; Wingfield *et al*. 1989; Gryzenhout *et al*. 2006b; Nakabonge *et al*. 2006). The devastating nature of the disease caused by *Chr. cubensis* and *Chr. austroafricana* has been an important motivating factor in the development and deployment of hybrid and clonal Eucalyptus forestry in South America and South Africa (Wingfield 2003).

Chrysoporthe cubensis has been known in Africa since the middle of the last century. The pathogen was first reported in 1952, as *Endothia eugeniae*, causing dieback of clove trees (*Syzygium aromaticum*) in Zanzibar (Nutman and Roberts 1952). On *Eucalyptus* spp., the fungus was first found in the Republic of Congo and thought to represent *Cryphonectria havanensis* (Hodges *et al*. 1985), but it was later identified as *Chr. cubensis* (Micales *et al*. 1987; Gryzenhout *et al*. 2004). *Chrysoporthe cubensis* has also been reported to cause disease of young *E. urophylla* in Cameroon (Gibson 1981), of *E. grandis* and *E. urophylla* in the Republic of Congo (Roux *et al*. 2003) and of *Eucalyptus* spp. in Mozambique and Kenya (Nakabonge *et al*. 2006). A recent study by Nakabonge *et al*. (2007) has suggested that *Chr. cubensis* is an introduced pathogen to Africa, with some strains originating from South America and others from Asia (Roux *et al*. 2003; Gryzenhout *et al*. 2006b).

Chrysoporthe austroafricana is an economically important pathogen of commercially grown *Eucalyptus* spp. in Africa (Wingfield 2003; Gryzenhout *et al*. 2004; Nakabonge *et al*. 2006). In South Africa, the pathogen caused substantial damage to plantation forestry and was partially managed through the selection and planting of disease tolerant clones (Wingfield *et al*. 1989; Wingfield 2003). The recent discovery of *Chr. austroafricana* on native *Syzygium* trees and *Eucalyptus* spp. in Malawi, Mozambique, Zambia (Roux *et al*. 2005; Nakabonge *et al*. 2006), Namibia (Vermeulen *et al*. 2008) and South Africa (Heath *et al*. 2006) has changed views regarding its origin. Once considered an introduced pathogen to Africa (Wingfield *et al*. 1989; Van Heerden and Wingfield 2001), *Chr. austroafricana* is currently suggested to be native to Africa, where it has undergone a host shift (Slippers *et al*. 2005) from native to non-native trees on the continent (Heath *et al*. 2006).

In Zambia, *Chr. austroafricana* was reported on *Syzygium* trees and *Eucalyptus* spp. from Kitwe on the Copperbelt region (Roux *et al*. 2005; Nakabonge *et al*. 2006). Whether this and other species in the Cryphonectriaceae also occur in other regions of the country is not known. Plantation forestry is increasing in importance in Zambia, becoming one of the major sources of income and employment for the population (Zimba 2005). This has necessitated the need to fully understand the potential impact of diseases on forest trees in order to suggest disease management strategies in the country. For this, surveys were conducted in several locations to establish the occurrence of the Cryphonectriaceae on native *Syzygium* trees and on introduced *Eucalyptus* spp., and to characterise these fungi using DNA sequences, morphological characteristics and pathogenicity tests.

Materials and Methods

Collection of samples

Isolates used in this study were obtained from stem cankers on *Eucalyptus* spp. (Myrtales) and *Syzygium* spp. (Myrtales) during surveys conducted in different geographical areas in Zambia between 2007 and 2008 (Fig. 1). Fungal fruiting structures from stem cankers were first tested for a purple discoloration in 3% potassium hydroxide (KOH) to confirm the affiliation to the Cryphonectriaceae (Gryzenhout *et al*. 2006a). Isolations were made by lifting spore drops from fresh fruiting structures with a sterile needle and transferring these to 2% malt extract agar (MEA) [20 g/l agar and 15 g/l malt extract (Biolab, Midland, Johannesburg) containing 100 mg/l streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany)]. Single spore cultures were incubated at 25°C under florescent light for 10 days to obtain pure cultures and sufficient mycelium for DNA extraction. All isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited in the CBS (Centraalbureau voor Schimmelcultures, The Netherlands) and linked bark material in the National Collection of Fungi, Pretoria (PREM).

Fig. 1. Map of Zambia showing locations where *Chrysoporthe* species were collected. 1 *Chrysoporthe austroafricana* in Kitwe, Copperbelt Province (Nakabonge et al. 2006). 2 *Chr. syzygiicola* on *Syzygium guineense* in Samfya, Luapula Province. 3 *Chr. zambiensis* on *Eucalyptus grandis* in Kapweshi, Luapula Province.

DNA extraction

Isolates for DNA extraction were selected from each host, geographic area of Zambia and morphological group. Mycelium from actively growing cultures of isolates was harvested, separately placed in 1.5-ml sterile Eppendorf tubes, freeze-dried and ground to a fine powder using sterile metal beads on a Mixer Mill (Type MM 301, Retsch® tissue lyser; Retsch, Germany) for 2 min at 45 cycles/s. DNA were extracted and purified using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Möller *et al*. 1992) and concentrations were determined using a Nanodrop ND-1000 Spectrophotometer v. 3.6 (Thermo Fisher Scientific, Wilmington, USA). After extraction, DNA were used as a template for amplification with the polymerase chain reaction (PCR). Internal transcribed spacer (ITS) regions ITS1 and ITS2, and the conserved 5.8 S gene of the ribosomal DNA were amplified using primers ITS1 and ITS4 (White *et al*. 1990). The β-tubulin gene region was amplified using primers Bt1a with Bt1b and Bt2a with Bt2b (Glass and Donaldson 1995). PCR reaction mixtures were prepared in a total volume of 25 μl as described by Gryzenhout *et al*. (2009).

PCR products were visualised on 2% agarose gels containing ethidium bromide under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100-bp molecular weight marker (O' RangeRuler™ 100-bp DNA ladder) (Fermentas Life Sciences, USA). Prior to DNA sequencing, PCR products were purified using Centri-sep spin columns (Princeton Separations, Adelphina, NJ) containing Sephadex G-50 (Sigma Aldrich, Amersham Biosciences, Sweden) as outlined by the manufacturer.

DNA sequencing and phylogenetic analyses

Purified PCR products were used as template DNA for cycle sequencing reactions using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The same primers for PCR reactions were also used for sequencing reactions run by an ABI PRISM™ 3100 Autosequencer (Applied Biosystems). Sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Applied Biosystems) to obtain both the forward and reverse sequences for each isolate. Sequences were then matched and analysed using Sequence Navigator version 1.0.1 (Applied Biosystems). Additional sequences for comparison were obtained from Genbank as published by Gryzenhout et al. (2004, 2005). For the purpose of this study, sequence alignments were done using the online interface (http://align.bmr.kyushu-u.ac.jp/mafft/software/) of MAFFT v. 5.667 (Katoh *et al*. 2002) incorporating the G-INS-i alignment algorithm. Alignments were checked and adjusted manually if sequences were not satisfactorily aligned by MAFFT.

Phylogeny of aligned sequences was determined using PAUP* 4.0 (Swofford 2002). A partition homogeneity test (PHT) (Farris *et al*. 1994) was applied to the combined data of rDNA ITS and β-tubulin sequences prior to exclusion of uninformative sites, using 1,000 replicates, to ascertain whether they could be collectively analysed. All gaps were coded as missing data and characters were assigned equal weight. The Heuristic search option with random stepwise addition and tree bisection reconnection (TBR) was used as the swapping algorithm. The Mulpar option was in effect and branches collapsed if they equalled zero. Confidence levels of the branching points were determined using 1,000 bootstrap replicates and distribution of 1000 trees.

Modeltest version 3.04 (Posada and Crandall 1998) was used to determine the most appropriate nucleotide substitution model to be applied to the combined DNA sequence alignment and GTR+I+G was chosen from the Akaike Information Criterion (AIC). Following this, a neighbour-joining (NJ) analysis using the GTR+I+G substitution model (Yang 1994) was conducted in PAUP. Here, identical sites were removed proportionally to base frequencies estimated from all sites, and

rates of invariable sites assumed to follow a gamma distribution and ties were broken if encountered. For phylogenetic analyses, *Cryphonectria parasitica* and *C. japonica* were used as the outgroup taxa and treated as paraphyletic sister groups to the in-groups.

Morphological characteristics

Fungal fruiting structures were cut from bark specimens, rehydrated for 1 min in boiling water and mounted in Jung Tissue Freezing Medium (Leica Microsystems, Wetzlar, Germany). Thin sections (10 µm) were cut using a Leica CM 100 Freezing microtome (Leica Microsystems) and mounted on slides in 85% lactic acid. Both cross-sections and structures obtained from fruiting bodies were examined under a Zeiss Axioskop light microscope (Carl Zeiss, Jena, Germany) using differential interference contrast. Sixty measurements of conidia and conidiophores for all specimens were taken from fresh slides at ×1,000 magnification. These are presented as (min –)(average – standard deviation) – (average + standard deviation)(– max) µm. Morphological characteristics of the fungi from Zambia were then compared with those of *Chrysoporthe* spp. known to occur on *Eucalyptus* spp. and other hosts from other countries (Gryzenhout *et al*. 2009).

An in vitro growth study was conducted on two groups of unidentified isolates, designated Group One (CMW29928, CMW29930) and Group Two (CMW29940, CMW29941), and *Chr. austroafricana* isolates (CMW2113, CMW9328, CMW13975) from South Africa and Zambia (Table 1). Agar plugs (5 mm diameter) from 7-day-old, single conidial colonies of each isolate were placed at the centres of 2% MEA in 90-mm-diameter Petri dishes to determine the radial growth of the cultures. Four Petri dishes for each isolate were incubated in the dark at 5°C intervals of each of six temperature regimes ranging from 10 to 35°C. Isolate growth rates were determined daily after incubation by measuring radial growth (two readings, perpendicular to each other, per plate) using a ruler. The experiment was repeated and data were pooled for the analyses. Data on growth rates were then analysed using a one-way Student's t test at 95% level of confidence using the SAS JMP IN version 4.0.4., Academic PROCANOVA procedure (Statistical Discovery Software, SAS Institute, NC, USA). Colony colours of the growing cultures at 25°C were compared using the mycological colour charts of Rayner (1970).

Table 1. Isolates sequenced in this study and those used in pathogenicity tests and growth studies.

Species name	Isolate number[a]	Alternative isolate number[b]	Herbarium number[c]	Host	Origin	Collector	GenBank accession numbers[d]		
Chrysoporthe syzygiicola	CMW29940[e]	CBS124488	PREM60260	*S. guineense*	Zambia	D. Chungu & J. Roux	FJ655005	FJ805230	FJ805236
	CMW29941	CBS124489	PREM60263	*S. guineense*	Zambia	D. Chungu & J. Roux	FJ655006	FJ805231	FJ805237
	CMW29942	CBS124490	PREM60258	*S. guineense*	Zambia	D. Chungu & J. Roux	FJ655007	FJ805232	FJ805238
Chr. zambiensis	CMW29928[e]	CBS124503	PREM60262	*E. grandis*	Zambia	D. Chungu & J. Roux	FJ655002	FJ858709	FJ805233
	CMW29929	CBS124512	PREM60261	*E. grandis*	Zambia	D. Chungu & J. Roux	FJ655003	FJ858710	FJ805234
	CMW29930	CBS124502	PREM60259	*E. grandis*	Zambia	D. Chungu & J. Roux	FJ655004	FJ858711	FJ805235
Chr. austroafricana	CMW2113	CBS112916		*E. grandis*	South Africa	M.J. Wingfield			
	CMW9327	CBS115843		*T. granulosa*	South Africa	M.J. Wingfield			
	CMW13975			*S. guineense*	Zambia	G. Nakabonge & J. Roux			
	CMW13976			*E. grandis*	Zambia	G. Nakabonge & J. Roux			

Pathogenicity tests

The relative pathogenicity of *Chrysoporthe* spp. strains was determined in inoculation tests on *E. grandis* saplings (6 months old) in a greenhouse. Seven isolates, randomly selected among two isolate groups from Zambia and *Chr. austroafricana* from South Africa and Zambia, were used. For *Chr. austroafricana*, isolate CMW2113, that has been shown to be highly virulent on *Eucalyptus* spp. in South Africa (Roux *et al*. 2003), and two isolates from *Syzygium* (CMW13975) and *Eucalyptus* (CMW13976) trees in Zambia, were selected for the study. Prior to inoculation tests, isolates were grown on 2% MEA and maintained at 25°C under continuous near-fluorescent light for 5 days.

A total of 160 *E. grandis* saplings from clone TAG5 with 10–12 mm diameters were acclimatized to greenhouse conditions (~13 h daylight and ~11 h darkness, approximately 25°C) for 2 weeks. Ten trees were inoculated with each test strain and ten others with a sterile water agar plug to serve as control. A 5-mm-diameter cork borer was used to remove a disc of bark from saplings to expose the cambium, and a mycelial plug of equal size was taken from actively growing cultures and placed into the wounds with the mycelia facing the cambium.

Wounds were sealed with parafilm 'M' (American National Can™ Chicago, USA) to avoid desiccation. Lesion lengths were recorded 4 weeks after inoculation to determine the pathogenicity of the isolates. Re-isolations were made from lesions and fungi recovered were observed under a light-microscope and identified using conidial characteristics. The experiment was repeated and data were pooled for analyses.

Data were subjected to analyses of variance (ANOVA) using the SAS JMP IN version 4.0.4. Before analysis, arcsine square root transformation of data was made to provide for a normal distribution and stabilised error variance (Sokal and Rohlf 1995) due to heteroscedasticity of variances among samples.

Results

Collection of samples

A total of 43 isolates resembling species of *Chrysoporthe* were collected from *Eucalyptus* and *Syzygium* trees in two geographic locations in Zambia. These included 24 isolates each from a separate *E. grandis* tree growing in plantations in Kapweshi and 19 isolates from separate *S. guineense* trees growing in a native forest in Samfya (Fig. 1). Symptoms on *Eucalyptus* trees included cracking of the bark at the bases of the stems, while isolates from *Syzygium* trees were obtained from dying branches and stem cankers.

DNA sequencing and phylogenetic analyses

Amplification products for the DNA regions considered in this study were approximately 519 bp (ITS) and 925 bp (β-tubulin) in size. Blast searches for the obtained sequences in the NCBI (www.ncbi.nlm.nih.gov) database confirmed that isolates collected from *E. grandis* and *S. guineense* in Zambia represent species in the Cryphonectriaceae. Isolates showed closest similarity to *Chr. austroafricana*. All new sequences were deposited in GenBank (Table 1) and their alignment to other species in *Chrysoporthe* in TreeBase (S279).

A combined sequence dataset, comprising ITS and β-tubulin gene sequences, produced 1,444 aligned sequence characters of which 1,172 were constant, 83 parsimony uninformative, and 189 parsimony informative. Congruence of the rDNA ITS and β-tubulin datasets was not supported by the partition homogeneity test (PHT) ($P=0.03$). This was most probably due to the highly conserved nature of the rDNA ITS genes of *Chrysoporthe* spp. (Myburg *et al*. 2002; Gryzenhout *et al*. 2004) resulting in poor resolution of the terminal branches. This P-value was less than the conventionally accepted P-value of 0.05 required to combine data. However, several studies have accepted a P-value less than 0.05 and have further stated that the conventional P-value of 0.05 is conservative (Cunningham 1997; Darlu and Lecointre 2002; Dettman *et al*. 2003). Furthermore, similar topology of the phylograms obtained from rDNA ITS and β-tubulin genes, and the increased bootstrap support for the groups obtained using the combined dataset, suggested that the data of these genes could be considered collectively. Thus, DNA sequence alignments of the rDNA ITS and β-tubulin gene regions were combined, irrespective of the incongruence of their loci.

Phylograms obtained by maximum parsimony and NJ analyses were similar and that obtained by NJ was chosen for presentation (Fig. 2). The *Chrysoporthe* isolates in the phylogram generated from the combined sequence data set resided in eight sub-clades (1–8), clustering separately from the outgroup taxa represented by *C. parasitica* and *C. japonica* [base frequencies: $\pi A=0.2387$, $\pi C=0.2571$, $\pi G=0.2660$, $\pi T=0.2103$; substitution rates: A/C=1.3649, A/G=1.8944, A/T=1.7195, C/G =1.2097, C/T=4.4921, G/T=1.0000; proportion of invariable sites (I) =0.5615; gamma shape distribution parameter =0.6928]. Clades 3–8 represented known isolates of *Chrysoporthe* that included *Chr. austroafricana*, *Chr. doradensis*, *Chr. inopina*, *Chr. cubensis* (South America), *Chr. cubensis* (Asia) and *Chrysoporthella hodgesiana*. Each clade was strongly supported by bootstrap values of >80%. New isolates from Zambia (as opposed to the previous isolate from this country in clade 3) resided in clades one and two that were distinct from those representing known species. Clade 1 represented isolates of '*Chrysoporthe zambiensis*' from cankers on the stems of *E. grandis* and clade 2 represented isolates of '*Chr. syzygiicola*' from *S. guineense* (100% bootstrap support). The *Chrysoporthe* spp. from Zambia residing in clade 1 and 2 were most closely related to *Chr. austroafricana* (Fig. 2).

The *Chrysoporthe* isolates comprising the eight clades in the phylogenetic analyses could be distinguished by several different base pairs in the rDNA ITS and β-tubulin gene regions (Table 2). Twenty-four base pairs were different between *Chr. austroafricana* and the two new taxa from Zambia. The '*Chr. syzygiicola*' isolates from *S. guineense* in Zambia differed from those of *Chr. austroafricana* in nine base pairs and from the '*Chr. zambiensis*' Isolates from *E. grandis* in Zambia in five base pairs. The latter species differed from isolates of *Chr. austroafricana* in ten base pairs (Table 3).

Morphological comparisons

No teleomorph structures were observed on the specimens from Zambia. Conidiomata of the fungi found on *E. grandis* (Fig. 3a) and *S. guineense* (Fig. 3g) in Zambia were generally similar to those for the descriptions of *Chrysoporthe* spp. (Myburg *et al*. 2002; Gryzenhout *et al*. 2004, 2009), being fuscous black to brown and slightly immersed. Conidiomatal necks consisted of textura porrecta. Conidia and conidiophores were similar in size and shape. Various morphological features were, however, distinct from those of the described '*Chr. zambiensis*' and '*Chr. syzygiicola*'.

The most important features that differentiate the Zambian species from each other and from other *Chrysoporthe* spp. were conidiomatal shape and conidial tendril colour. Conidiomata of the '*Chr. zambiensis*' specimens from *E. grandis* (Group One) had more globose to ovoid bases with tapering necks and they were rust brown, while conidiomata of the '*Chr. syzygiicola*' specimens from *S. guineense* (Group Two) were darker (fuscous black to brown) and had a rostrate shape. These shapes were distinct from those of other *Chrysoporthe* spp. (Table 4). Conidia of the '*Chr. zambiensis*' from *E. grandis* were also exuded as pale luteous spore tendrils, similar to those of *Chr. doradensis*, but different from other *Chrysoporthe* spp. (Table 4).

Cultures of *Chr. austroafricana*, '*Chr. zambiensis*' and '*Chr. syzygiicola*' differed in growth rates on 2% MEA. The optimal temperature for growth was 30°C for '*Chr. zambiensis*' and '*Chr. syzygiicola*', and 25°C for *Chr. austroafricana*. The growth rate of '*Chr. zambiensis*' (6.3 mm/day) was more rapid than '*Chr. syzygiicola*' (5.4 mm/day) with isolates in this group covering the 90-mm plates in 7 days rather than the 8 days for the latter. These were slower growing than *Chr. austroafricana* cultures (8.0 mm/day), which covered the same size plates in 5 days (Fig. 5). The differences in growth rates among these isolates of *Chrysoporthe* were statistically significant ($P<0.05$). None of the isolates grew at 10°C or 35°C on 2% MEA.

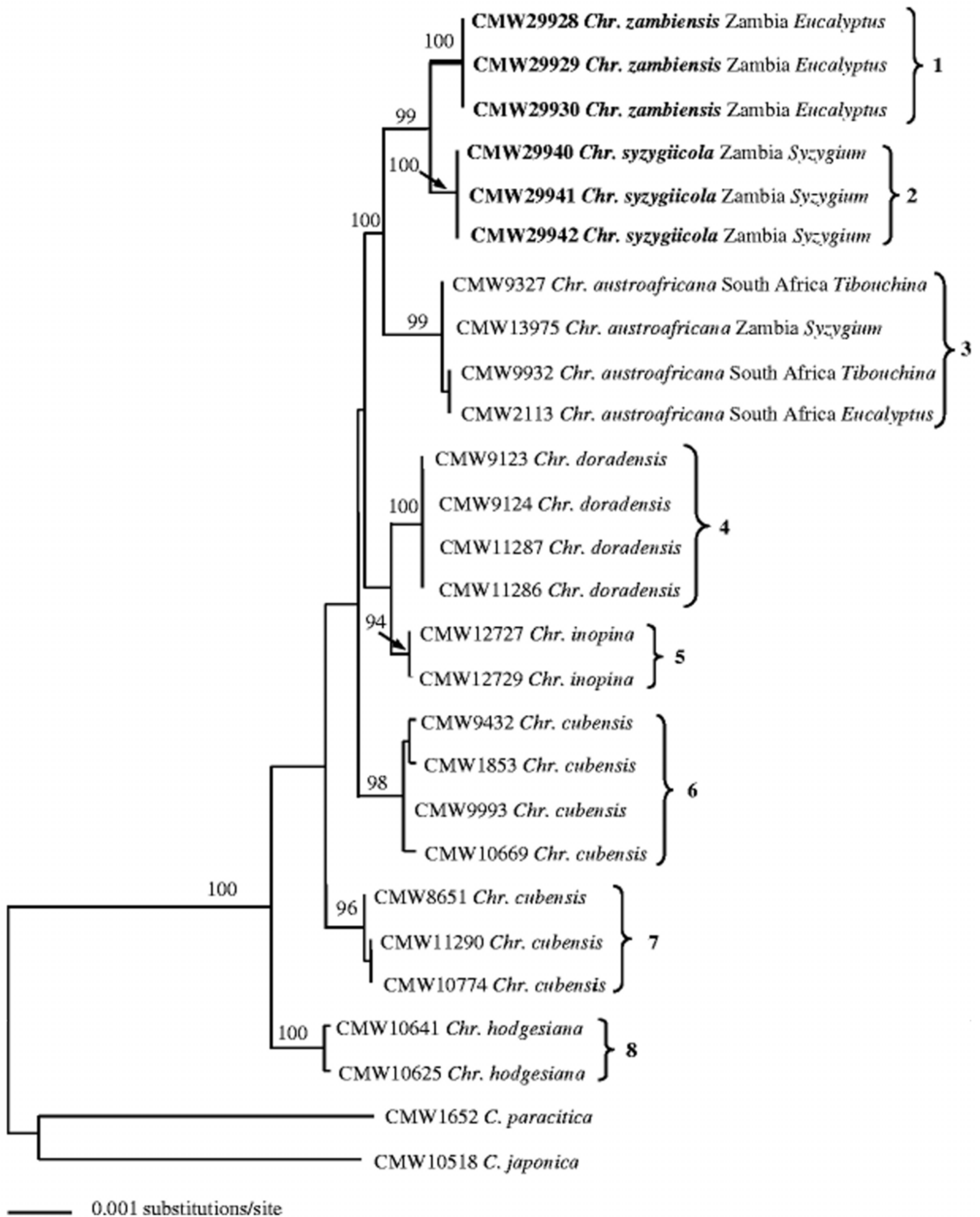

Fig. 2. Neighbour-joining phylogram of the Cryphonectriaceae, indicating the phylogenetic placement of *Chrysoporthe zambiensis* and *Chr. syzygiicola*, obtained from distance analysis of combined ITS and β-tubulin data using the GTR+I+G DNA substitution model (gamma shape distribution parameter =0.6928). Bootstrap values of 1,000 replicates above 80% are indicated on the branches. Isolates sequenced in this study are in bold. *Cryphonectria parasitica* and *C. japonica* sequences were used as outgroup.

Table 2. Summary of polymorphic nucleotides found within sequences of the ribosomal ITS region and two regions in the β-tubulin genes for all known *Chrysoporthe* species, including *Chr. zambiensis* and *Chr. syzygiicola* described in this study. Polymorphic nucleotides unique to each species are shown in bold typeface.

Species	Beta-tubulin 1 (Bt1a/Bt1b)																		
	34	127	171	174	178	179	181	184	201	205	219	237	239	278	296	347	350	374	401
Chrysoporthe austroafricana	G	C	C	C	A	G	C	C	A	C	C	C	-	C	C	C	G	C	T
Chr. syzygiicola	C	C	C	T	G	G	C	C	A	C	C	C	-	C	C	A	T	C	T
Chr. zambiensis	C	C	C	T	G	G	C	C	A	C	C	C	-	C	C	C	G	C	A
Chr. cubensis[a]	C	T	C	C	G	A	A	A	A	T	T	T	-	T	T	C	G	C	T
Chr. cubensis[b]	C	C	C	C	G	G	A	A	A	T	T	T	-	T	C	C	G	C	T
Chr. doradensis	C	C	T	C	A	G	A	A	A	T	C	C	-	T	C	C	G	A	T
Chr. inopina	C	C	C	C	G	G	A	A	A	T	C	C	T	C	C	C	G	C	T
Chrysoporthe hodgesiana	C	C	C	C	G	G	A	A	G	T	T	T	-	C	T	C	G	C	T

Species	ITS1/5.8S/ITS2										Beta-tubulin 2 (Bt2a/Bt2b)														
	88	118	151	350	403	411	429	436	440	505	80	97	105	148	149	203	209	238	243	269	353	374	383	422	464
Chrysoporthe austroafricana	-	A	C	C	-	T	A	A	C	G	T	G	C	T	G	C	C	G	C	A	C	T	C	G	C
Chr. syzygiicola	-	A	C	-	-	C	A	A	C	G	T	G	C	T	G	C	A	G	G	A	C	T	C	G	C
Chr. zambiensis	-	A	C	-	-	C	A	A	A	G	T	G	C	T	G	A	A	G	G	A	C	T	C	G	C
Chr. cubensis[a]	-	A	T	-	-	C	A	A	C	G	C	G	C	T	G	C	C	G	G	A	C	T	C	G	A
Chr. cubensis[b]	-	A	C	-	T	T	G	G	C	A	C	G	C	C	G	C	C	T	G	A	T	T	T	G	C
Chr. doradensis	T	A	C	-	-	C	A	A	C	G	C	G	T	C	G	C	T	G	G	G	T	T	C	G	C
Chr. inopina	-	G	C	-	-	C	A	G	C	G	C	G	T	C	A	C	T	G	G	A	T	T	C	G	C
Chrysoporthe hodgesiana	-	G	C	-	-	C	A	G	C	G	C	A	C	T	G	C	C	G	G	A	C	C	C	A	C

Table 3. Number of fixed DNA base pairs between different phylogenetic groups of *Chr. austroafricana*, *Chr. syzygiicola* and *Chr. zambiensis*.

Groups	*Chrysoporthe zambiensis*	*Chrysoporthe syzygiicola*
Chrysoporthe austroafricana	10	9
Chrysoporthe syzygiicola	5	

Table 4. Comparison of *Chrysoporthe zambiensis* and *Chrysoporthe syzygiicola* with other *Chrysoporthe* species.

Species	Pathogenicity on *Eucalyptus*	Optimal temp. for growth	Growth rate on MEA mm/d	Conidiomata base width (μm)	Coniodiomata shape	Max. neck length (μm)	Teleomorph	Conidium shape	Conidium size (μm)	Spore mass	Reference
Chr. austroafricana	High[a]	25–30°C	8.0	80–120	Pyriform to pulvinate	200	Known	Oblong to ovoid	3–4(–4.5)× 1.5–2	Bright yellow	This study; Gryzenhout et al. 2004
Chr. syzygiicola	Very high[a]	30°C	5.4	250–500	Ovoid with tapering neck	220	Unknown	Oblong	2.1–)2.5–3.5(–4.0) ×(1.2-)1.5–2.0	Bright yellow	This study
Chr. zambiensis	Low[a]	30°C	6.3	208–310	Pyriform to rostrate	300	Unknown	Oblong	(2.5–)3.0–3.5(–4.0) ×(1.1–)1.5–2.0	Pale cream	This study
Chr. doradensis	Low[b]	30°C	-	100–290	Pyriform to pulvinate	300	Known	Oblong to ovoid to cylindrical	(3–)3.5–5(–6.5) ×1.5–2(–2.5)	Pale cream	Gryzenhout et al. 2005
Chr. inopina	Unknown	25°C	-	70–710	Subulate to pyrifom to pulvinate	780	Known	Oblong	(3–)3.5–4× (1.5–) 2–2.5	Bright yellow	Gryzenhout et al. 2006b
Chr. hodgesiana	Low[b]	25°C	-	145–635	Pulvinate, occasionally pyrifom	380	Unknown	Oblong	(3–)3.5–5(–5.5)× 1.5–2(–2.5)	Bright yellow	Wingfield et al. 2001; Gryzenhout et al. 2004
Chr. cubensis	Low[a]	30°C	-	100–950	Pyriform to pulvinate	230	Known	Oblong	(3–)3.5× 4.5(–5)	Bright yellow	Roux et al. 2003; Gryzenhout et al. 2004

[a] In the greenhouse

[b] In the field; species described in this study are in bold

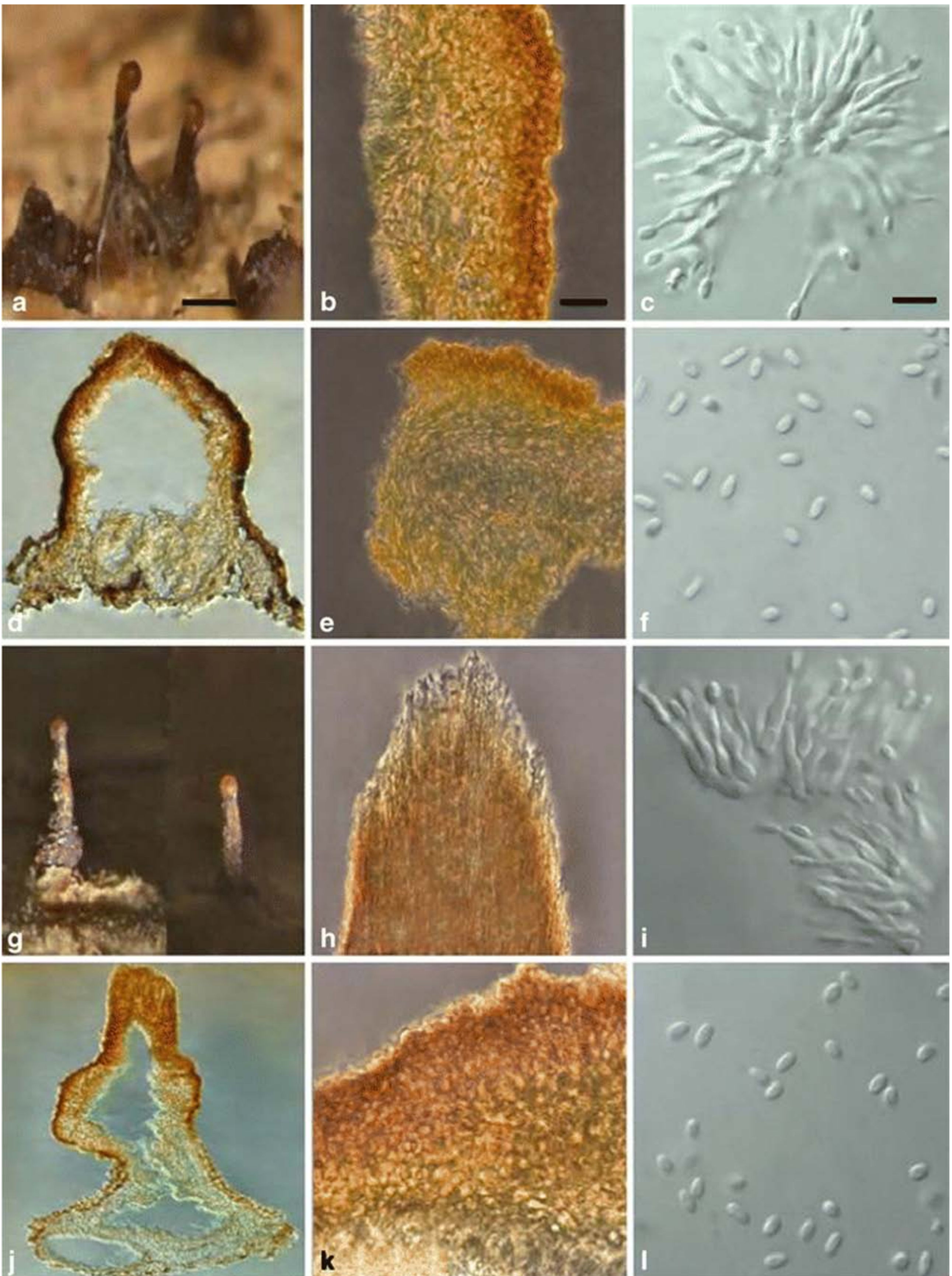

Fig. 3. Fruiting structures of **a–f** *Chrysoporthe zambiensis*. **a** Conidiomata on bark. **b** Tissue of conidiomatal neck. **c** Conidiophores with attached conidiogenous cells. **d** Longitudinal section through conidiomata. **e** Tissue of conidiomatal base. **f** Conidia. **g-l** Chrysoporthe syzygiicola. g Conidiomata on bark. **h** Tissue of conidiomatal neck. **i** Conidiophores with attached conidiogenous cells. **j** Longitudinal section through conidiomata. **k** Tissue of conidiomatal base. **l** Conidia. Scale

bars **a**,**d**,**g**,**j** 100 µm; **b**,**e**,**h**,**k** 20 µm; **c**,**f**,**i**,**l** 10 µm

Taxonomy

Morphological characteristics and phylogenetic data provided good evidence that the fungi from *E. grandis* and *S. guineense* in Zambia represent undescribed species of *Chrysoporthe*. These species were distinct in culture, having growth rates that were significantly different from their closest relative, *Chr. austroafricana*. Conidiomatal shape and tendril colour were also different from those of other species of *Chrysoporthe*. Phylogenetic analyses revealed significant differences in DNA sequence data for the isolates from Zambia, compared to the described species of *Chrysoporthe*. Two new species are thus described to accommodate the isolates from Zambia.

Specimens linked to the descriptions of the two new species have only anamorph structures. Based on DNA sequence data and morphology they have, however, been firmly established as two species of *Chrysoporthe*. In the absence of a teleomorph, the anamorph genus *Chrysoporthella*, described for *Chrysoporthella hodgesiana* (Gryzenhout *et al*. 2004), would be appropriate to accommodate the Zambian species. However, the name *Chrysoporthella hodgesiana* has caused considerable confusion where scientists have failed to appreciate the connection of this species to *Chrysoporthe*, or have mistakenly believed that this species is the anamorph of *Chr. cubensis*. As *Chrysoporthe* is a recently established genus (Gryzenhout *et al*. 2004), and includes only five species (Gryzenhout *et al*. 2009), application of a formal anamorphic genus is confusing and unnecessary, even where only anamorph structures are found. For the same reason, no anamorph genera have been established for other recently described genera in the Cryphonectriaceae (Gryzenhout *et al*. 2009).

In the absence of the teleomorph, Recommendation 59A.3 of the International Code of Botanical Nomenclature (McNeill *et al*. 2007) is followed. Here, it is stated that the description of an anamorph name of the holomorph is unnecessary if it is proven that the anamorph structures represent the holomorph, and there is no functional need for a second name. The Code also states, in Art. 59.7, that if teleomorph structures are found at a later stage, the teleomorph structures can be epitypified to the existing name that was based on anamorph structures, to preserve its status. We, therefore, describe the two species from Zambia in *Chrysoporthe*, even though sexual structures are not present.

Chrysoporthe zambiensis Chungu, Gryzenh. & Jol. Roux, sp. nov.
Figs. 3a–f & 4a, MycoBank MB509677.

Description: Conidiomata ferruginea rostrata globosa. Conidia oblonga. Massulae sporarum laete luteae. Culturae albae maculis cinnamomeis, ad 6.3 mm/day in temperatura optima 30°C crescentes.

Conidiomata slightly immersed, rust brown, rostrate, globose, of *Chrysoporthella*-type, conidiomatal bases above the bark surface 110–240 µm high, 208–310 µm wide with necks 250–300 µm. Conidiomatal locules are uniloculate with convoluted inner surfaces, locules 150–285 µm diameter (Fig. 3a, d). Neck tissue of textura porrecta (Fig. 3b), base tissue of textura globulosa, the outer cells with thickened walls (Fig. 3e). Conidiophores hyaline, with basal cells of irregular shape, (2.2–)3.0–4.5(–6.5) × (1.1–)2.0–2.0(–3.5) µm, 95% confidence intervals = 3.91–4.44 × 2.50–2.94 µm, branched irregularly at the bases or above into cylindrical cells with or without separating septa, total length of conidiophores (14.2–)15.2–16.0(–17.5) µm, 95% confidence intervals = 13.79–16.02 µm (Fig. 3c). Conidiogenous cells phialidic, apical or lateral on branches beneath a septum, cylindrical with or without attenuated apices, (1.2–)2.0–3.5(–5.4) µm wide, 95% confidence intervals = 3.01–3.43 µm wide (Fig. 3c). Conidia hyaline, non-septate, oblong, (2.5–)3.0–3.5(–4.0) × (1.1–)1.5–2.0 µm, 95% confidence intervals = 3.17–3.23 × 1.58–1.62 µm (Fig. 3f). Spore masses pale luteous (cream).

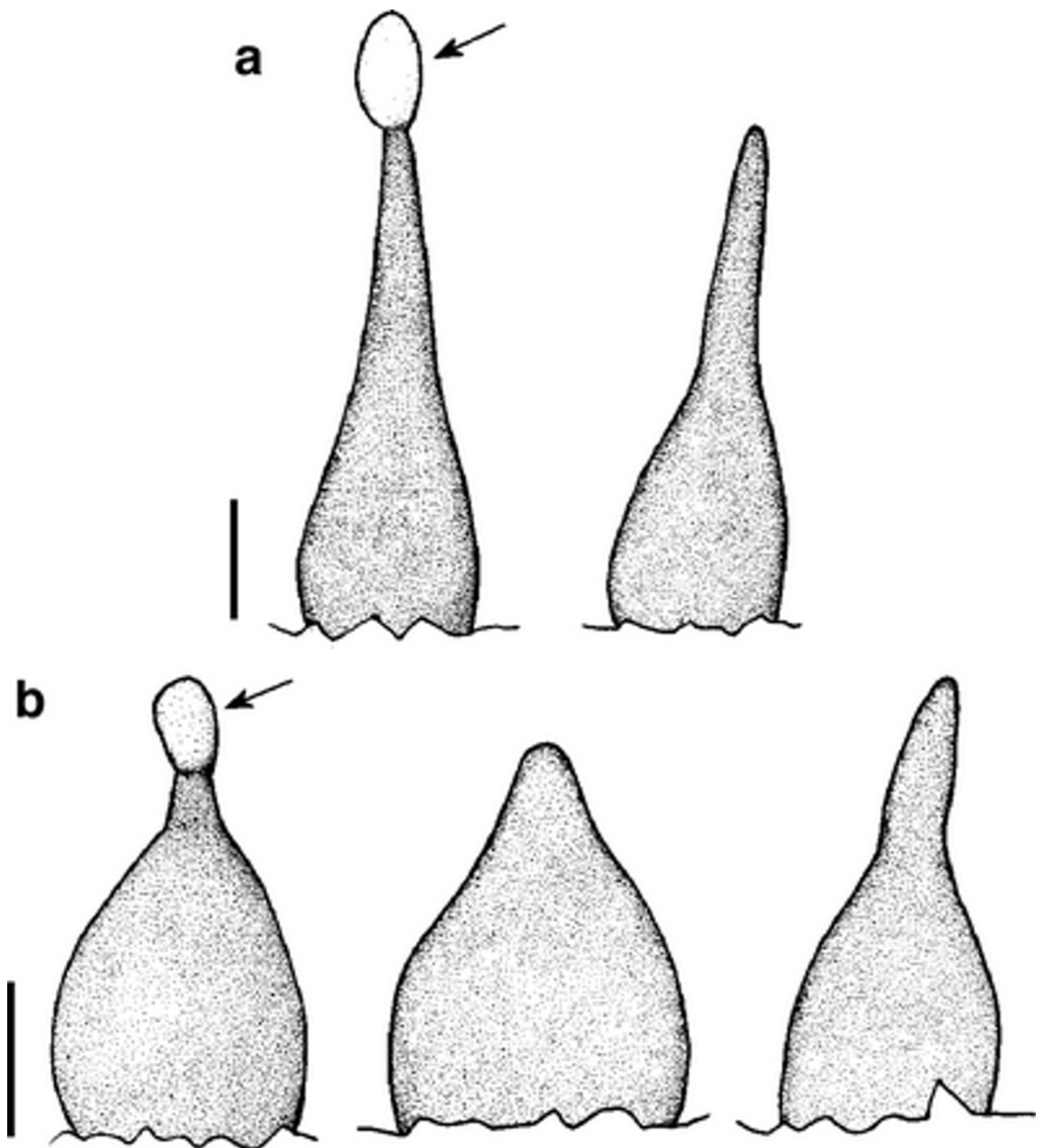

Fig. 4. Schematic drawings of diagnostic characteristics of: a *Chrysoporthe zambiensis* and b *Chrysoporthe syzygiicola* conidiomata (spore drops indicated with arrows). Scale bars 100 µm.

Etymology: Name refers to Zambia, the country where the fungus was first collected.

Teleomorph: Not seen.

Culture characteristics: On MEA, *Chr. zambiensis* cultures white with cinnamon patches, fluffy with a smooth margin, growing fast at the rate of 6.3 mm/day at 30°C optimal temperature covering a 90-mm plate in 7 days (Fig. 5). *Chrysoporthe zambiensis* does not sporulate in culture after sub-culturing and does not produce teleomorph structures in culture.

Substrate: Stems of *Eucalyptus grandis* trees.

Distribution: Kapweshi, Luapula Province, Zambia.

Specimens examined: ZAMBIA, Luapula Province, Kapweshi, on stems of *Eucalyptus grandis*, collected August 2008, D. Chungu & J. Roux, holotype PREM60262, living ex-type culture CMW29928/CBS124503, paratype PREM60261 and PREM60259, living ex-paratype cultures CMW29929/CBS124512 and CMW29930/CBS124502.

Chrysoporthe syzygiicola Chungu, Gryzenh. & Jol. Roux sp. nov.
Figs. 3g–l & 4b, MycoBank MB509660

Distribution: Conidiomata globosa fusco-nigra vel brunnea. Conidia oblonga vel ovoidea. Massulae sporarum vivide flavissimae vel luteae. Culturae albae medio maculis umbrinis vel avellaneis, ad 5.4 mm/day in temperatura optima 30°C crescentes.
Conidiomata slightly immersed in bark, globose, fuscous-black to brown, of *Chrysoporthella*-type,, conidiomatal bases above the bark surface 197–234 µm high, 250–500 µm wide with necks 150–220 µm long. Conidiomata uniloculate, locules 290–361 µm diameter with convoluted inner surfaces (Fig. 3g, j). Neck tissue of textura porrecta (Fig. 3h), basal tissue of textura globulosa, the outer cells with thickened walls (Fig. 3k). Conidiophores hyaline, with basal cells of irregular shape, (2.0–)3.0–5(–7.5) × (1.3–)2.5–3.0(–4.5) µm, 95% confidence intervals = 4.51–4.89 × 2.80–3.11 µm, branched irregularly at the bases or above into cylindrical cells with or without separating septa, total lengths of conidiophores (10.9–)11.1–12.9(–13.7) µm, 95% confidence intervals = 11.90–12.51 µm (Fig. 3i). Conidiogenous cells phialidic, apical or lateral on branches beneath a septum, cylindrical shape with or without attenuated apices, (1.4–)1.8–2.9(–4.5) µm wide, 95% confidence intervals = 2.62–2.99 µm wide, collarettes and periclinal thickenings inconspicuous (Fig. 3i). Conidia hyaline, non-septate, oblong to ovoid (2.0–)2.5–3.5(–4.0) × (1.2–)1.5–2.0 µm, 95% confidence intervals = 3.08–3.12 × 1.68–1.72 µm (Fig. 3l). Spore masses bright yellow luteous.

Etymology: Name refers to the *Syzygium* trees hosting this fungus, where cola means dweller in Latin.

Teleomorph: Not seen.

Culture characteristics: On MEA, *Chr. syzygiicola* cultures white with umber to hazel patches in the middle, fluffy with a smooth margin, growing fast at the rate of 5.4 mm/day at 30°C optimal temperature covering a 90-mm plate in 8 days. *Chrysoporthe syzygiicola* does not sporulate in culture after sub-culturing and does not produce teleomorph structures in culture.

Substrate: Stems of *Syzygium guineense*.

Distribution: Samfya, Luapula Province, Zambia.

Specimens examined: ZAMBIA, Luapula Province, Samfya, on stem of *Syzygium guinense*, collected August 2008, D. Chungu & J. Roux, holotype PREM60260, living ex-type culture CMW29940/CBS124488, paratype PREM60263 and PREM60258, living ex-paratype cultures CMW29941/CBS124489 and CMW29942/CBS124490.

Chrysoporthe hodgesiana (Gryzenh. & M.J. Wingf.) Chungu, Gryzenh. & M.J. Wingf. comb. nov.
MycoBank MB515496

Basionym: *Chrysoporthella hodgesiana* Gryzenh. & M.J. Wingf. Studies in Mycology 50: 135–136.

Notes: For reasons stated above, the name *Chrysoporthella hodgesiana* is confusing in a small and

well-known genus such as *Chrysoporthe*. There is also no practical need to refer to this species using a name other than *Chrysoporthe*, because the holomorph of the fungus is well established. We, therefore, propose that the species should be renamed as *Chrysoporthe hodgesiana* (Gryzenh. & M.J. Wingf.) Chungu, Gryzenh. & M.J. Wingf. to encourage the use of a one name system for *Chrysoporthe*.

Pathogenicity tests

Cambial lesions, as well as external bark discolouration, were found on all inoculated Eucalyptus saplings at the time of data collection. No lesions developed in any of the control inoculations. The analyses of variance revealed significant differences between strains of the *Chrysoporthe* spp. ($P < 0.001$) (Fig. 5). The most pathogenic isolates were those of *Chr. syzygiicola* (Group Two), which produced average lesion lengths of 160 mm. *Chr. zambiensis* (Group One) produced average lesions lengths of 60.5 mm. The three isolates of *Chr. austroafricana* showed considerable variation in lesion length, with CMW2113 more pathogenic (110 mm) than CMW13975 (78 mm). The shortest lesions (9.55 mm), that were not significantly different from the controls, were produced by *Chr. austroafricana* isolate CMW13976 from Zambia. *Chrysoporthe zambiensis* and *Chr. syzygiicola* were consistently isolated from lesions on inoculated trees, confirming that they were the cause of the lesions, while no *Chrysoporthe* spp. were obtained from the control inoculations.

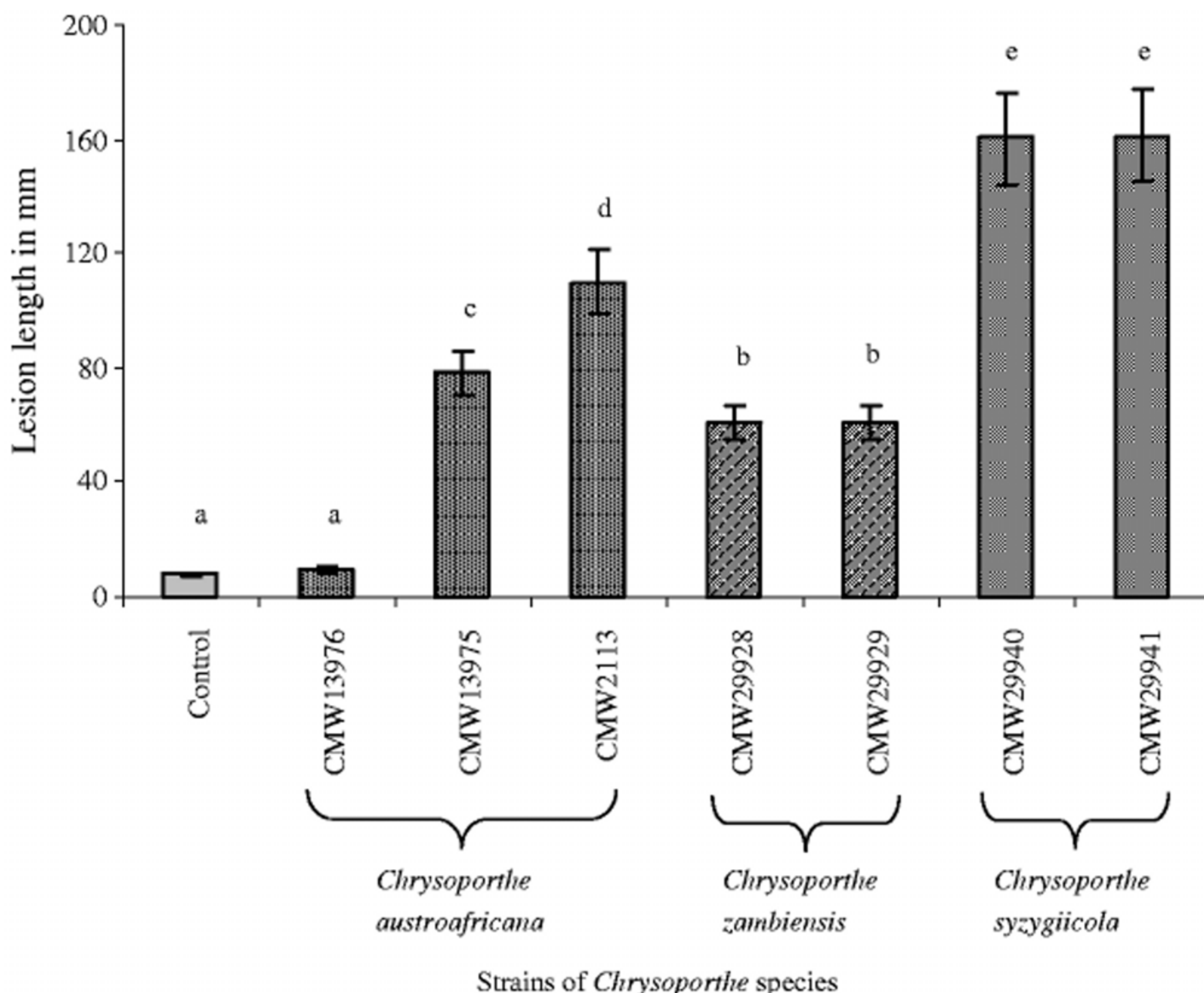

Fig. 5. Lesion lengths in mm associated with inoculations of isolates of *Chrysoporthe austroafricana* (CMW13976, CMW13975, CMW2113), *Chrysoporthe zambiensis* (CMW29928, CMW29929) and *Chrysoporthe syzygiicola* (CMW29940, CMW29941) on a *Eucalyptus grandis* clone in the greenhouse after 4 weeks. A total of 160 lesion lengths were measured. Different letters

above the bars indicate significant differences in the pathogenicity of the *Chrysoporthe* strains ($P < 0.001$); error bars indicate the standard error of the means.

Discussion

In this study, we have shown that unknown isolates of *Chrysoporthe* obtained from E. grandis and S. guineense in Zambia represent two undescribed species, different from other Chrysoporthe spp. The description of these new taxa as Chr. zambiensis and Chr. syzygiicola is supported by morphological characteristics and DNA sequence data. These species were both shown to be pathogenic to the Eucalyptus clone tested in the greenhouse, with Chr. syzygiicola being highly pathogenic.

The two newly described *Chrysoporthe* spp. from Zambia have morphological characteristics typical of *Chrysoporthe*. *Chrysoporthe zambiensis* and *Chr. syzygiicola* have fuscous black to rust brown conidiomata that are slightly immersed. Similarities also exist in the tissue of the conidiomatal necks and bases that were similar to those in previous descriptions of *Chrysoporthe* spp. No statistical differences were observed in the conidial sizes or shapes of these species from other *Chrysoporthe* spp. (Gryzenhout *et al*. 2009), all producing oblong, hyaline and non-septate conidia.

Based on phylogenetic analyses and morphology, *Chr. zambiensis* and *Chr. syzygiicola* are most closely related to *Chr. austroafricana*. *Chrysoporthe austroafricana* is common in southern Africa and has previously also been reported from Zambia (Nakabonge *et al*. 2006). However, conidiomata of *Chr. zambiensis* had more globose to ovoid bases with tapering necks and were rust brown, while conidiomata of *Chr. syzygiicola* were darker and had a more rostrate shape. These shapes were distinct from other *Chrysoporthe* spp., including *Chr. austroafricana*. Furthermore, *Chr. syzygiicola* has conidia that are expelled as pale luteous spore drops while *Chr. austroafricana* and *Chr. zambiensis* produced bright yellow spore masses. *Chrysoporthe zambiensis* and *Chr. syzygiicola* also differ from *Chr. austroafricana* in their higher optimal growth temperatures at 30°C, compared to that of *Chr. austroafricana* (25°C).

In this study, we report on two new species of *Chrysoporthe* for which only the anamorph is known. This is similar to *Chrysoporthella hodgesiana*, which was described in a new anamorph genus because no sexual structures were known for it (Gryzenhout *et al*. 2004). The absence of sexual structures would normally have required that the two species from Zambia be described in the anamorphic genus *Chrysoporthella*. However, use of two names for *Chrysoporthe* spp., where some species have the anamorph name and others the teleomorph name, would have been extremely confusing, and we therefore chose to describe these fungi in *Chrysoporthe*. For the same reason, the new combination *Chrysoporthe hodgesiana* has been provided in an attempt to establish a more uniform and logical taxonomy for this genus.

Symptoms on *Eucalyptus* trees from which *Chr. zambiensis* was collected were characterised by swelling cankers and cracking at tree bases. Both *Chr. zambiensis* and *Chr. syzygiicola* produced significant lesions on 6-month-old trees inoculated in a greenhouse, with some trees inoculated with *Chr. syzygiicola* starting to die after 4 weeks. Although *Chr. syzygiicola* was found only on *Syzygium* trees, our findings clearly show that this is a potentially important pathogen of *Eucalyptus* spp. Nonetheless, the pathogenicity tests for *Chr. syzygiicola* and *Chr. zambiensis* on *S. guineense* were not investigated due to unavailability in South Africa of this native Zambian tree species. Such tests, which are needed in future, would have provided valuable information relating to the importance of these fungi to the natural environment of Zambia.

The relative susceptibility of *Eucalyptus* spp. to stem canker disease induced by different *Chrysoporthe* spp. has been shown in a number of studies in which *Chr. austroafricana*, *Chr. cubensis*, *Chr. doradensis* and *Chr. hodgesiana* were used to inoculate trees (Table 4). Following inoculation, *Chr. austroafricana* from South Africa was shown to be the most pathogenic species (Roux *et al*. 2003; Wingfield 2003; Rodas *et al*. 2005; Gryzenhout *et al*. 2005). Results of the present study have, however, shown that *Chr. zambiensis* and *Chr. syzygiicola* are more pathogenic on Eucalyptus than both isolates of *Chr. austroafricana* from Zambia and South Africa, including isolate CMW 2113 from South Africa that has been selected for its high level of pathogenicity and which was included in previous studies (Roux *et al*. 2003; Rodas *et al*. 2005). This suggests that *Chr. syzygiicola* and *Chr. zambiensis* are more pathogenic than *Chr. austroafricana* and that both species, particularly *Chr. syzygiicola* because of significantly greater lesions induced in this study, should be considered as potentially important pathogens of *Eucalyptus* in the region.

Non-native *Eucalyptus* plantations in Zambia are often established in proximity of closely related native tree species in the Myrtales (Chidumayo 1997; Chipeta 1999). In such cases, pathogens of either these related hosts could cause cross-infection that may result in serious disease outbreaks (Slippers *et al*. 2005). *Chrysoporthe austroafricana* in South Africa was first found on introduced *Eucalyptus* spp. and only years later on native *Syzygium* sp., considered as a native host (Heath *et al*. 2006). Because pathogenic fungi and related diseases of native trees in Zambia have been less studied, and diseases of *Eucalyptus* plantations have only recently received attention (Shakachite 1991; Roux *et al*. 2005; Muimba-Kankolongo *et al*. 2009), threats from these fungi for closely related non-native trees deserve further investigations.

Introduced pathogens can infect and cause considerable damage to native trees. Examples of this include chestnut blight caused by *C. parasitica* (Anagnostakis 1987; Heiniger and Rigling 1994), and Dutch Elm Disease caused by *Ophiostoma ulmi* and *O. novo-ulmi* (Sinclair and Lyon 2005). Similarly, *Chr. syzygiicola* and *Chr. zambiensis* could be destructive if introduced into other countries or continents where *Eucalyptus* or other related and susceptible trees grow as natives. In this regard, countries such as Australia, with large numbers of native *Eucalyptus* spp., and countries with native trees also possibly susceptible, such as those in South and Central America with high diversities of native Melastomataceae, should be particularly concerned regarding the possible spread of these pathogens. Similarly, *Chrysoporthe* spp. present in Zambia might pose an important threat to the biodiversity of other countries in Africa with native Myrtaceae or *Eucalyptus* plantations.

Results of this study have important implications for *Eucalyptus* plantation forestry in Zambia and in the sub-region. Tree breeding programmes should take *Chr. zambiensis* and *Chr syzygiicola* into consideration to ensure eucalypts that are selected for plantation development are not susceptible to infection by these fungi. Likewise, *Chr. austroafricana* that is also present in Zambia and in other countries in southern Africa should be part of assessments for resistance in breeding programmes. Furthermore, *Chr. cubensis* that occurs in the Democratic Republic of Congo, Malawi and Mozambique (Roux *et al*. 2003; Nakabonge *et al*. 2006) has the potential to cause significant damage to *Eucalyptus* forestry in Zambia and other countries of the sub-region.

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