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Endophytic Cryphonectriaceae on native Myrtales: possible origin of Chrysoporthe canker on plantation-grown *Eucalyptus*

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ABSTRACT

Chrysoporthe austroafricana (Cryphonectriaceae) is a damaging canker pathogen on *Eucalyptus* species in Southern Africa. Recent studies have shown that the fungus occurs on native *Syzygium* species and that it has apparently undergone a host range expansion from these native trees to infect non-native *Eucalyptus*. The aim of this study was to consider whether *C. austroafricana* and other Cryphonectriaceae might exist as endophytes in native Myrtaceae, providing a source of inoculum to infect non-native Myrtales. Healthy branches were collected from Myrtaceae in Mozambique, incubated in florist foam, allowed to dry gradually and monitored for the appearance of fruiting bodies resembling species in the Cryphonectriaceae. Isolates were identified based on DNA sequence data. Two species in the Cryphonectriaceae were obtained, representing the first evidence that species in the Cryphonectriaceae occur as endophytes on native Myrtales, thus providing a source of inoculum to infect non-native and susceptible trees. This has important implications regarding the movement of planting stock used by ornamental tree and forestry enterprises.

Key words: canker pathogens; forestry; Melastomataceae; Myrtaceae; plants for planting; quarantine

1.0 INTRODUCTION

Fungi in the Cryphonectriaceae include a number of important tree pathogens globally, both in native and commercial plantation ecosystems (Gryzenhout *et al*. 2009). The best known of these is *Cryphonectria parasitica*, the cause of chestnut blight, that has lead to the near extinction of American and European chestnut trees in their respective native ranges (Anagnostakis 1987). Related species in the genus *Chrysoporthe* (previously known as species of *Cryphonectria*) gained notoriety in the 1970's when they were identified as important pathogens of commercially grown *Eucalyptus* species in Brazil (Hodges *et al*. 1976; Wingfield 2003).

Chrysoporthe (Cryphonectriaceae) includes a number of important eucalypt pathogens (Gryzenhout *et al.* 2009; Wingfield 2003), including *C. austroafricana* in Africa (Gryzenhout *et al.* 2004; Nakabonge *et al*. 2006), *C. cubensis* in Latin America (Gryzenhout *et al*. 2004; Hodges *et al*. 1976) and Africa (Roux and Apetorgbor 2010), and *C. deuterocubensis* in Asia (Sharma *et al*. 1985; Van der Merwe *et al*. 2010) and Africa (Nakabonge *et al*. 2007). Infections by *Chrysoporthe* species result in stem and root collar cankers after colonization of the bark, cambium and woody tissues at the bases of *Eucalyptus* trees (Hodges *et al*. 1976; Sharma *et al*. 1985; Wingfield *et al* 1989). Infection of young trees results in death, while stem cankers on older trees make the stems prone to wind breakage (Nakabonge *et al.* 2006; Sharma *et al*. 1985; Wingfield 2003).

Chrysoporthe species have a host range restricted to plants in the family Myrtales. Host genera include *Lagerstroemia* (Gryzenhout *et al*. 2006), *Miconia* (Rodas *et al*. 2005), *Psidium* (Hodges 1988), *Syzygium* (Hodges *et al*. 1986), *Tibouchina* (Wingfield *et al*. 2001) and a number of others (Barreto *et al.* 2006; Gryzenhout *et al*. 2006; Seixas *et al*. 2004). In most countries where *Eucalyptus* species are grown as non-natives, they occur in close proximity to related, native plants in the Myrtales (Seixas *et al*. 2004; Wingfield *et al*. 2001). The occurrence of similar fungal species on both the native and non-native hosts suggests that some *Chrysoporthe* species have undergone host shifts (Slippers *et al*. 2005) from the native Myrtales, eg. *Miconia*, *Syzygium*, *Tibouchina* species, to infect non-native *Eucalyptus* spp. (Heath *et al*. 2006, Van der Merwe *et al.* 2010, 2012). Evidence from population genetic studies suggests that *C. cubensis* is native to Latin America (Gryzenhout *et al*. 2009), where it underwent a host shift from native Myrtales to infect non-native *Eucalyptus* species (Van der Merwe *et al*. 2012). Similarly, *C. austroafricana* is an African fungus that has undergone a host shift from native African Myrtales (Heath *et al*. 2006) to infect Australian *Eucalyptus* species grown as non-natives in plantations.

At least two of the *Eucalyptus* pathogens, *C. cubensis* and *C. deuterocubensis* have moved beyond their purported regions of origin. *Chrysoporthe cubensis*, believed to be native in South and Central America, has been found in Central and West Africa (Gibson 1981; Roux *et al*. 2003; Roux and Apetorgbor 2010). Likewise, *C. deuterocubensis,* which is believed to be native to Asia (Myburg *et al*. 2002; Pegg *et al*. 2010; van der Merwe *et al*. 2010), has been found in East and Southern Africa (Nakabonge *et al*. 2006; Van der Merwe *et al*. 2010). These important pathogens have been recorded only from non-native *Eucalyptus* species and *S. aromaticum* (clove) in

Africa. The limited distribution of *C. deuterocubensis* outside East Africa, together with a low population diversity (Nakabonge *et al*. 2007), strongly supports the hypothesis that it was introduced to the African continent, most likely from Asia with the trade in cloves (Gryzenhout *et al.* 2006; Roux *et al*. 2003).

The accidental movement of fungi to new environments, and the disease epidemics that have subsequently arisen in some cases, has raised increasing concern as the incidence and impact of these introductions has increased (Brasier 2008; Desprez-Loustau *et al.* 2007; Liebhold *et al.* 2012; Wingfield *et al*. 2015). The trade in life plants, sometimes also referred to as "plants for planting", and timber have been identified as two of the main pathways of pathogen introductions into new regions (Brasier 2008; Liebhold *et al*. 2012). It has for example been suggested that the chestnut blight pathogen, *C. parasitica*, was introduced into the United States of America with living plants (Dutech *et al*. 2012; Milgroom *et al*. 1992), while the most likely route of movement of *Chrysoporthe* species is still not well understood. A pathway of spread that has not received attention for fungi in the Cryphonectriaceae, is where they might have been carried as symptomless endophytes. This would be in seemingly healthy plants or commercially traded plant tissue such as that used for floral arrangements.

Endophytes are microorganisms living within plant tissues, for all or part of their life cycle, without causing any apparent or detectable symptoms of disease (Arnold *et al*. 2003; Bacon and White 2000; Petrini *et al*. 1993). These organisms can be latent or opportunistic pathogens, causing disease when infected plants are exposed to unsuitable environmental conditions (Bacon and White 2000). Some endophytic microorganisms have also been reported to benefit their host plants by providing protection from herbivores or insect infestation (Arnold and Lewis 2005; Clay 1986; Siegel *et al*. 1985), by enhancing growth (Ren *et al*. 2011), improving drought tolerance (Hubbard *et al*. 2012) and protection against pathogens (Arnold *et al*. 2003). Endophytes probably occur in all plant species and plant parts (Rosenblueth and Martínez Romero 2006; Sturz *et al*. 2000) and while they contribute significantly to the hyper-diversity of fungi, they typically go unnoticed (Arnold 2008; Hawksworth 2001).

Despite the fact that *Chrysoporthe* species are important pathogens of *Eucalyptus* species, very little is known regarding their origin or how they have emerged as important pathogens on non-native, commercially propagated trees. The fact that *C. austroafricana* is found sporulating on bark and dead branches of native Myrtaceae in areas where the fungus occurs as a pathogen of *Eucalyptus* suggests that the fungus and its relatives possibly could occur as non-damaging endophytes in asymptomatic trees. The aim of this study was to test this hypothesis by making isolations from asymptomatic tissues of Myrtales growing in a native environment and to identify the resulting fungi. Because fungi in the Cryphonectriaceae are likely to develop and sporulate gradually as plant tissue dies, a novel technique to detect possible infections by them was applied.

2.0 MATERIALS AND METHODS

2.1 Endophyte isolations

During the course of two field surveys in Mozambique in July 2010 and August 2011, segments (~30cm length, ~1cm diameter) were cut from healthy branches of various native and non-native Myrtales in eucalypt-growing areas of the country. All leaves were removed from the samples at the time of collection. Trees sampled included native species of *Dissotis* and *Syzygium*, *Eugenia capensis*, and non-native *Psidium guajava* in the Central, Northern and Southern Provinces of Mozambique (Table 1). A total of 89 trees, collected in six provinces of Mozambique, were sampled. Six trees were from Inhambane (*Syzygium guineense*), seven were from Gaza (four of *E. capensis* and three of *Syzygium cordatum*), 23 from Nampula (20 *S. guineense* and three *S. cordatum*), ten from Niassa (six *Dissotis* sp., two *S. cordatum* and two *S. guineense*), ten from Sofala (one *P. guajava*, two *S. cordatum*, three *S. guineense* and four *Syzygium* sp.) and 33 trees from Zambézia (two *Dissotis* sp., two *P. guajava*, 13 *S. cordatum*, 16 *S. guineense* (Table 1). The numbers and species of trees sampled were dependent on their availability, since in most areas, native trees have been burned and felled for replacement with agricultural crops.

Branch segments were placed in individual brown paper bags, which were sealed in larger plastic bags to retain moisture, and transported to the laboratory. All the branch samples were surface-disinfested with 70% ethanol for 1 min to remove epiphytes and then placed in moist chambers to induce the growth and sporulation of species in the Cryphonectriaceae from within and below the bark. Moist chambers consisted of moistened florist foam in square plastic containers, or 1.5 liter plastic bottles, moistened with a small quantity of water at the base of the container to prevent the plant material from drying out inordinately rapidly. Branch samples were inserted into the florist foam and the containers were placed in a greenhouse at 25 °C, with natural day-night lighting. The lids were removed from the cake savers and plastic bottles as keeping them on resulted in too much moisture accumulation on the plant material. Branch samples were monitored weekly for the presence of fruiting structures resembling those of the Cryphonectriaceae, and to ensure that a moist environment was maintained. All branch samples were monitored over a two month period.

Where fungal fruiting bodies were present, single spore drops were transferred, using a sterile needle, to 2% Malt Extract Agar (MEA) including 20 g/L of agar (Biolab, Midland, Johannesburg) and 15 g/L malt with 100 mg/L streptomycin sulphate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and incubated at 25 °C until the onset of fungal growth. For some fungi, fruiting bodies were cut open using a sharp, sterile scalpel blade, and the exposed spore masses transferred to sterile 2% MEA. Where no fresh spore drops were visible, plant tissue was incubated in moist chambers to induce spore production. Pure cultures of all isolates obtained in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2 Identification of isolates

Fungi obtained from branch samples were identified based on morphological characteristics and comparisons of DNA sequence data. All putative Cryphonectriaceae isolated from different species of the Myrtales, and from different geographic regions, were selected for identification based on DNA sequencing (Table 1).

2.2.1 DNA extraction

For DNA extraction, mycelium was harvested from actively growing cultures of isolates resembling species in the Cryphonectriaceae and placed in 1.5 ml sterile Eppendorf tubes and freeze dried overnight. Mycelium was then ground to a fine powder using sterile metal beads on a Mixer Mill (Type MM 301, Retsch® tissue lyser, Retsch, Germany) for two minutes at 30 cycles per second. DNA was extracted and purified using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Möller *et al*. (1992). The nucleic acids were then pelleted using centrifugation (2800 rpm for two minutes) and washed in 70% ethanol, followed by suspension in sterilized distilled water. Two microliters of RNaseA (10 µg/µl) were added to each tube and incubated at room temperature for 24 hours to digest residual RNA. The concentrations of the extracted DNA samples were determined using a Nanodrop ND-1000 Spectrophotometer v.3.6 (Thermo Fisher Scientific, Wilmington, USA).

2.2.2 PCR amplification and purification

The polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS1, ITS2) regions, including the 5.8 S gene of the ribosomal RNA (rRNA) operon, with the primer pair ITS1 and ITS4 (White *et al*. 1990) for all isolates. Depending on identities based on the ITS and 5.8S sequence results, sequence data were also obtained for the β-tubulin 1 and β-tubulin 2 regions (BT) with primers BT1a/ BT1b, BT2a/ BT2b (Glass and Donaldson 1995). The PCR reaction mixtures used to amplify the different loci consisted of 2.5 units FastStart *Taq* polymerase

(Roche Applied Science, USA), 1× PCR buffer, 1–1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 µm of each primer and approximately 50–100 ng of fungal genomic DNA, made up to a total reaction volume of 25 µl with sterile de-ionised water. The amplification conditions included an initial denaturation of the double stranded DNA at 96 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, annealing for 1 min at 54 °C to 56 °C (depending on the primer), extension for 90 s at 72 °C and a final elongation step of 10 min at 72 °C. The PCR amplification products were separated by electrophoresis on 2% agarose gels stained with GelRed in a TAE buffer and visualized under UV light. Amplified fragments were purified using Centri-sep mini spin columns (Princeton Separations, Adelphina, HJ) containing 6% Sephadex G-50 (Sigma, Steinhein, Germany) following the manufacturer's instructions.

2.2.3 DNA sequencing and phylogenetic analyses

The purified PCR products were used as template DNA for cycle sequencing reactions using an Icycler thermal Cycler to generate sequences in both the forward and reverse directions with the same primers used for the PCR reactions, in 10 µl PCR mixtures. The composition of the mixture was 2 µl of Sabax water, 2 µl ready reaction buffer (BigDye), 1 µl of 5 x reaction buffer, 1 µl primer (10 mM) and 4 µl of the PCR product. The BigDye terminator sequencing kit v3.1 (Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems, USA) were used for sequencing reactions.

Sequences for isolates obtained in this study were compared against the data base of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) to obtain an indication of their identities. For all isolates residing in in the Cryphonectriaceae, additional sequences for comparison were obtained from Genbank (http://www.ncbi.nlm.nih.gov) and TreeBASE (http://www.treebase.org) and combined into datasets for further analyses (Table 2). Sequence alignments were made 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. When sequences were not satisfactorily aligned by MAFFT, alignments were checked and adjusted manually.

PAUP* 4.0 (Swofford 2002) was used to determine the phylogeny of aligned sequences. Sequence data sets for the ITS and BT regions were first analyzed separately, and then in combined analyses. Before combining sequence data sets, a partition homogeneity test (PHT) (Farris *et al*. 1994) was conducted to determine whether the data sets could be combined. For the analyses, combined data of rDNA ITS and BT sequences were examined prior to exclusion of uninformative sites, using 1000 replicates, to ascertain whether they could be collectively analyzed. All gaps were coded as missing data and characters were assigned equal weight. Maximum Parsimony (MP) analyses were done in PAUP 4.0 (Swofford 2002). 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 equaled zero. Confidence levels of the branching points were determined using 1000 bootstrap replicates and distribution of 1000 trees.

For phylogenetic analyses, *Cryphonectria parasitica*, which was defined as a paraphyletic sister group to the in-group taxa, was chosen as the out-group taxon. For the parsimony analyses, the tree length (TL), retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI) were determined.

3.0 RESULTS

3.1 Endophyte isolations

Fungal fruiting bodies resembling species in the Cryphonectriaceae began to appear on branch samples after two months of incubation, and as samples gradually dried in the florist foam. Yellow to orange tinged stromata and ascomatal and pycnidial necks, exuding mostly orange to yellow spore masses, were found breaking through the bark of branches. Isolations were made directly from these spore masses. Isolates resembling species of Cryphonectriaceae were obtained from native *Dissotis* sp. in Niassa Province, *S. cordatum* and *S. guineense* in Inhambane, Gaza, Nampula, Niassa, Sofala and Zambézia Provinces. Isolates of this group of fungi were also obtained from non-native *P. guajava* in the Sofala Province. In total, putative Cryphonectriaceae isolates were obtained from 35 of the 89 branch samples considered in this study.

3.2 Identification of isolates

DNA was obtained from all isolates tentatively identified as of the Cryphonectriaceae and sequence products of ~600 bp were obtained for the ITS regions and ~500 bp for the BT regions. Blast searches with these sequences suggested the presence of two genera amongst the isolates. The combined dataset for the ITS and BT gene regions had a total length of 1007 characters. From this dataset, 700 characters were excluded and 307 characters were parsimony-informative. The partition homogeneity test of the combined regions conducted in PAUP resulted in a P-value of 0.01, thus lower than the conventionally accepted value of 0.05 required to combine data. However, several studies have accepted P-values greater than 0.001 (Cunningham 1997; Dettman *et al*. 2003) and a decision was made to do so in this case. The parsimony analysis of the combined dataset resulted in the retention of 91 most parsimonious trees (TL = 316, CI = 0.854, RI = 0.968, RC = 0.827, HI = 0.146).

Isolates collected in this study grouped with *Chrysoporthe austroafricana* and *Celoporthe woodiana* (Figure 1). For *Chr. austroafricana*, three isolates were obtained in Niassa Province from *Dissotis* and *S. cordatum*; 11 isolates were obtained in Nampula Province, from *S. cordatum* and *S. guineense*; 11 isolates were obtained in Zambézia Province, from *S. cordatum* and *S. guineense*; five isolates were obtained in Sofala Province, from *P. guajava*, *S. cordatum*, *S. guineense* and one unknown *Syzygium* sp.; four isolates were obtained in Inhambane Province, from *S. guineense*. For *Cel. woodiana* only one isolate was collected and this was from *S. cordatum* from Xai-Xai in the Gaza Province (Table 1).

4.0 DISCUSSION

The results of this study show conclusively, and for the first time, that members of the Cryphonectriaceae exist as endophytes in host plants. This discovery is important because it provides an explanation for many recent and surprising outbreaks of cankers caused by the Cryphonectriaceae, particularly in South America, Southern

Africa and South East Asia (Gryzenhout *et al*. 2004; Heath *et al*. 2006; Myburg *et al*. 2002; Nakabonge *et al*. 2006; Roux *et al*. 2005). Where pathogens residing in this group of fungi have emerged as apparently non-native, for example *C. cubensis* in Africa (Gibson 1981, Myburg *et al*. 2002, Roux *et al*. 2003), it now seems likely that the fungus would have been introduced into that area on plant material, such as that of *Eucalyptus,* used in plantation development, or on ornamental Myrtales brought into the region.

It has previously been speculated that members of the Cryphonectriaceae in Africa might originate from endophytic infections. However, attempts to isolate these fungi as endophytes by Vermeulen *et al*. (2011) were not successful. This might be explained by the fact that isolations on agar could have been overgrown by more rapidly developing fungi such as for example the Botryosphaeriaceae, which are common endophytes in Angiosperm trees including the Myrtales (Pavlic *et al*. 2007; Roux *et al*. 2000, 2001; Smith *et al*. 1996b; Slippers and Wingfield 2007). In addition, endophytic infections are obviously not uniformly found across plant (in this case branch) tissue and isolations from small pieces of tissue could easily not have included the Cryphonectriaceae.

A unique, but simple aspect of this study was the technique used to determine the possible presence of the Cryphonectriaceae in branch samples. Here, we attempted to simulate a slow drying of the branch samples, as might occur on broken branches falling to the understory in forests. The notion to use this approach arose from an observation (Wingfield M.J unpublished) of abundant fruiting structures of the Cryphonectriaceae on branches of *Tibouchina* species in Colombia, where these trees are commonly infected by various *Chrysoporthe* species (Gryzenhout *et al*. 2004; Rodas *et al*. 2005; Wingfield *et al*. 2001). Thus, placing branch samples with considerable surface area into moistened florist foam, allowing the samples to dry out slowly over a number of months, stimulated the Cryhonectriaceae present to develop and sporulate. This would also explain previous observations where the Cryphonectriaceae have often be found sporulating on branch stubs and dead bark of native trees (Vermeulen *et al*. 2011).

Two species of Cryphonectriaceae were discovered as endophytes on Myrtales in this study. Of these, *C. austroafricana* has previously been reported from *Eucalyptus* species in Mozambique (Nakabonge *et al*. 2006; Roux *et al*. 2005) and is most likely native to Africa (Heath *et al*. 2005, 2006; Nakabonge *et al*. 2006; Vermeulen *et al*. 2011). *Chrysoporthe austroafricana* is also known to occur on *Eucalyptus* spp. in Malawi, Mozambique, South Africa and Zambia (Nakabonge *et al*. 2006; Roux *et al*. 2005), non-native *Tibouchina* in South Africa (Myburg *et al*. 2002) and native *Syzygium* in South Africa (Heath *et al*. 2006), Zambia (Chungu *et al*. 2010) and Namibia (Vermeulen *et al*. 2011).

The discovery of *C. austroafricana* as a endophyte on native *Dissotis*, *S. cordatum* and *S. guineense* in this study provides added evidence that this fungus is native to Africa. It is unlikely that it is a serious pathogen on these native plants but importantly, it is able to move to non-native and commercially important plants such as *Eucalyptus*, and to cause serious disease problems (Gryzenhout *et al.* 2009; Old *et al.* 2003; Pegg *et al.* 2010; Wingfield *et al.* 1989, 1997).

The second species of Cryphonectriaceae found in this study was represented by only a single isolate. This was tentatively identified as *Cel. woodiana*, although there were sufficient sequence differences to suggest that it might represent a unique species. *Celoporthe woodiana* is a recently described species, which prior to the present study, was known only from the non-native garden-tree *Tibouchina granulosa* in South Africa (Vermeulen *et al*. 2011). It has, however, also been shown to infect *Syzygium* and *Eucalyptus* species in artificial inoculation studies (Vermeulen *et al*. 2011). The fact that this fungus was found as an endophyte of a native *Syzygium* sp. could suggest that it is native to Southern Africa, and like *C. austroafricana*, could easily emerge as an important pathogen of *Eucalyptus* species in the region.

Many species of Myrtales are planted as non-natives globally and they include some of the most important commercially propagated plantation trees as well as popular ornamentals. The discovery that the Cryphonectriaceae, including some of the most important and damaging pathogens of trees (Gryzenhout *et al*. 2009), exist as endophytes in native trees, suggests strongly that infected plant material, such as rooted or even unrooted cuttings has been, and continues to be, a source of movement of these pathogens. Clearly, the appearance of these fungi as serious pathogens has arisen from native and apparently non-damaging fungi undergoing host shifts to infect non-native and susceptible commercially propagated *Eucalyptus* species in South and Central America, Africa and Asia.

The nursery/plant trade has been, and continues to, actively move seeds, seedlings and plants of the Myrtales internationally (Ferreira *et al*. 2008; Old *et al*. 2003; Paine *et al*. 2011). Species of *Chrysoporthe* have already been shown to be capable of host shifts between native and non-native Myrtales (Heath *et al*. 2006; Van der Merwe *et al.* 2010, 2012) and it is likely that there are many more examples of this situation. Many "new encounter" diseases, both of economic and ecological importance can thus be expected in future (Wingfield *et al.* 2010). A greater effort must obviously be made to contain the movement of life plants, eg. "Plants for planting", as has been suggested by numerous tree health specialists globally (eg. Montesclaros Declaration www.iufro.org/science/divisions/division-7/70000/publications/montesclaros-declaration/).

The cross-border movement of living plants represents one of the most important sources of the introduction of plant pathogens into new regions (Liebhold *et al*. 2012) and there have been recent calls for new strategies to limit this threat (Wingfield *et al*. 2015). The discovery of two species, in two genera of Cryphonectriaceae, as endophytes represents a significant breakthrough in better understanding the pathways of spread of this important group of fungal tree pathogens. The often cryptic nature of endophytes, especially pathogens, makes them of important quarantine concern, since they can move undetected in plant material.

The discovery of members of the Cryphonectriaceae as endophytes in healthy plant tissue raises intriguing questions beyond those species occurring on the Myrtales in the southern Hemisphere. These fungi reside in two very distinct clades, with the genera *Cryphonectria* dominant on the Fagales in the northern hemisphere, and *Chrysoporthe* dominant on the Myrtales in the southern Hemisphere (Gryzenhout *et al*. 2009). It seems probable that important northern hemisphere tree pathogens such as *C. parasitica* would exist as endophytes on trees in for example Asia where it is native (Milgroom *et al*. 1992, 1996). Existence in this niche would then provide a very plausible means by which *C. parasitica* moved easily on rooted cuttings of the Fagaceae into Europe and North America in the early 1900's. This is an important hypothesis relating to the global movement of tree pathogens that urgently needs testing. The technique described in this study provides an alternative method to detect slower growing endophytes of trees and specifically to obtain cultures needed for pathology and other related studies.

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Table 1. Number of Myrtalean trees sampled and number of trees from which endophytic *Cryphonectriaceae* were obtained in Mozambique

Host ID	**Geographic origin**	**Number of trees sampled per host species**	**Number of trees with Cryphonectriaceae isolated**	**Identity of Cryphonectriaceae species obtained**
Dissotis sp.	Niassa, Lichinga	6	2	*Chrysoporthe austroafricana*
	Zambézia, Gurué	2	0	
Eugenia capensis	Gaza, Zongoene	4	0	
Psidium guajava	Sofala, Galinha	1	1	*Chrysoporthe austroafricana*
	Zambézia, Gurué	2	0	
Syzygium cordatum	Gaza, Zongoene	3	1	*Celoporthe woodiana*
	Nampula, Ilha de Mozambique	3	2	*Chr. austroafricana*
	Niassa, Lichinga	2	1	*Chr. austroafricana*

	Sofala, Galinha	2	1	*Chr. austroafricana*
	Zambézia, Gurué	13	6	*Chrysoporthe austroafricana*
S. guineense	Inhambane, Inhambane	3	3	*Chrysoporthe austroafricana*
	Inhambane, Inharrime	3	1	*Chrysoporthe austroafricana*
	Nampula, Ilha de Mozambique	14	6	*Chrysoporthe austroafricana*
	Nampula, Ribáuè	6	3	*Chrysoporthe austroafricana*
	Niassa, Lichinga	2	0	
	Sofala, Galinha	3	1	*Chrysoporthe austroafricana*
	Zambézia, Gurué	16	5	*Chrysoporthe austroafricana*
Syzygium sp.	Sofala, Galinha	4	2	*Chrysoporthe austroafricana*
Total		89	35	

Table 2. Sequences obtained in this study and from GenBank.

Species name	**Isolate no.** [a]	**Host**	**Origin**	**GenBank accession no.**[b]
Celoporthe dispersa	CMW9978	*Syzygium cordatum*	South Africa	AY214316, DQ267135, DQ267141
Cel. dispersa	CMW9976	*S. cordatum*	South Africa	DQ267130, DQ267136, DQ267142
Cel. eucalypti	CMW26911	*Eucalyptus* EC48 clone	China	HQ730838, HQ730818, HQ730828
Cel. eucalypti	CMW26913	*Eucalyptus* EC48 clone	China	HQ730839, HQ730819, HQ730829
Cel. guangdongensis	CMW12750	*Eucalyptus* sp.	China	HQ730830, HQ730810, HQ730820
Cel. indonesiensis	CMW10779	*S. aromaticum*	Indonesia	AY084007, AY84031, AY084007
Cel. indonesiensis	CMW10780	*S. aromaticum*	Indonesia	AY084008, AY084032, AY084008
Cel. syzygii	CMW24912	*Syzygium* sp.	China	HQ730833, HQ730813, HQ730823
Cel. syzygii	CMW24914	*Syzygium* sp.	China	HQ730834, HQ730814, HQ730824
Cel. woodiana	CMW13936	*Tibouchina granulosa*	South Africa	DQ267131, DQ267137, DQ267143
Cel. woodiana	CMW13937	*T. granulosa*	South Africa	DQ267132, DQ267138, DQ267144
Cel. woodiana[c]	CMW37246	*S. cordatum*	Zongoene, Gaza, Mozambique	JX842758, JX842770, JX842764
Cel. fontana	CMW29375	*S. guineense*	Zambia	GU726940, GU726952,

Cel. fontana	CMW29376	*S. guineense*	Zambia	GU726941, GU726953,
Chrysoporthe austroafricana	CMW2113	*Eucalyptus grandis*	South Africa	AF046892, AF273067, AF273462
C. austroafricana	CMW9327	*T. granulosa*	South Africa	AF273473, AF273060, AF273455
C. austroafricana[c]	CMW36297	*S. cordatum*	Ilha de Moçambique, Mozambique	JX842754, JX842766, JX842760
C. austroafricana[c]	CMW37557	*S. guineense*	Galinha, Sofala, Mozambique	JX842753, JX842765, JX842759
C. austroafricana[c]	CMW37563	*Dissotis* sp.	Lichinga, Niassa, Mozambique	JX842757, JX842769, JX842763
C. austroafricana[c]	CMW37564	*Dissotis* sp.	Lichinga, Niassa, Mozambique	JX842756, JX842768, JX842762
C. austroafricana[c]	CMW37566	*S. guineense*	Lichinga, Niassa, Mozambique	JX842755, JX842767, JX842761
C. cubensis	CMW1853	*S. aromaticum*	Brazil	AF046891, AF273070, AF273465
C. cubensis	CMW10778	*S. aromaticum*	Brazil	AY084006, AY084030, AY084018
C. deuterocubensis	CMW8650	*S. aromaticum*	Indonesia	AY084001, AY084024, AY084013
C. deuterocubensis	CMW8651	*S. aromaticum*	Indonesia	AY084002, AY084014, AY084026
C. doradensis	CMW11286	*E. grandis*	Ecuador	AY214289, AY214217, AY214253
C. doradensis	CMW11287	*E. grandis*	Ecuador	AY214290, AY214218, AY214254
C. hodgesiana	CMW10625	*Miconia theaezans*	Colombia	AY956970, AY956979, AY956980

C. hodgesiana	CMW10641	*T. semidecandra*	Colombia	AY692322, AY692326, AY692325
C. inopina	CMW12727	*T. lepidota*	Colombia	DQ368777, DQ368806, DQ368807
C. inopina	CMW12729	*T. lepidota*	Colombia	DQ368778, DQ368808, DQ368809
C. syzygiicola	CMW29940	*S. guineense*	Zambia	FJ655005, FJ805230, FJ805236
C. syzygiicola	CMW29941	*S. guineense*	Zambia	FJ655006, FJ805231, FJ805237
C. zambiensis	CMW29928	*E. grandis*	Zambia	FJ655002, FJ858709, FJ805233
C. zambiensis	CMW29929	*E. grandis*	Zambia	FJ655003, FJ858710, FJ805234
Cryphonectria parasitica	CMW7048	*Quercus virginiana*	Japan	AF368330, AF273076, AF273470
Cryphonectria parasitica	CMW13749	*Castanea mollisima*	Japan	AY697927, AY697943, AY697944

[a]CMW refers to Culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

[b]Accession numbers refers to sequence data of the ITS, ß-tubulin 1 and ß-tubulin 2 gene regions

[c]Isolates obtained in this study

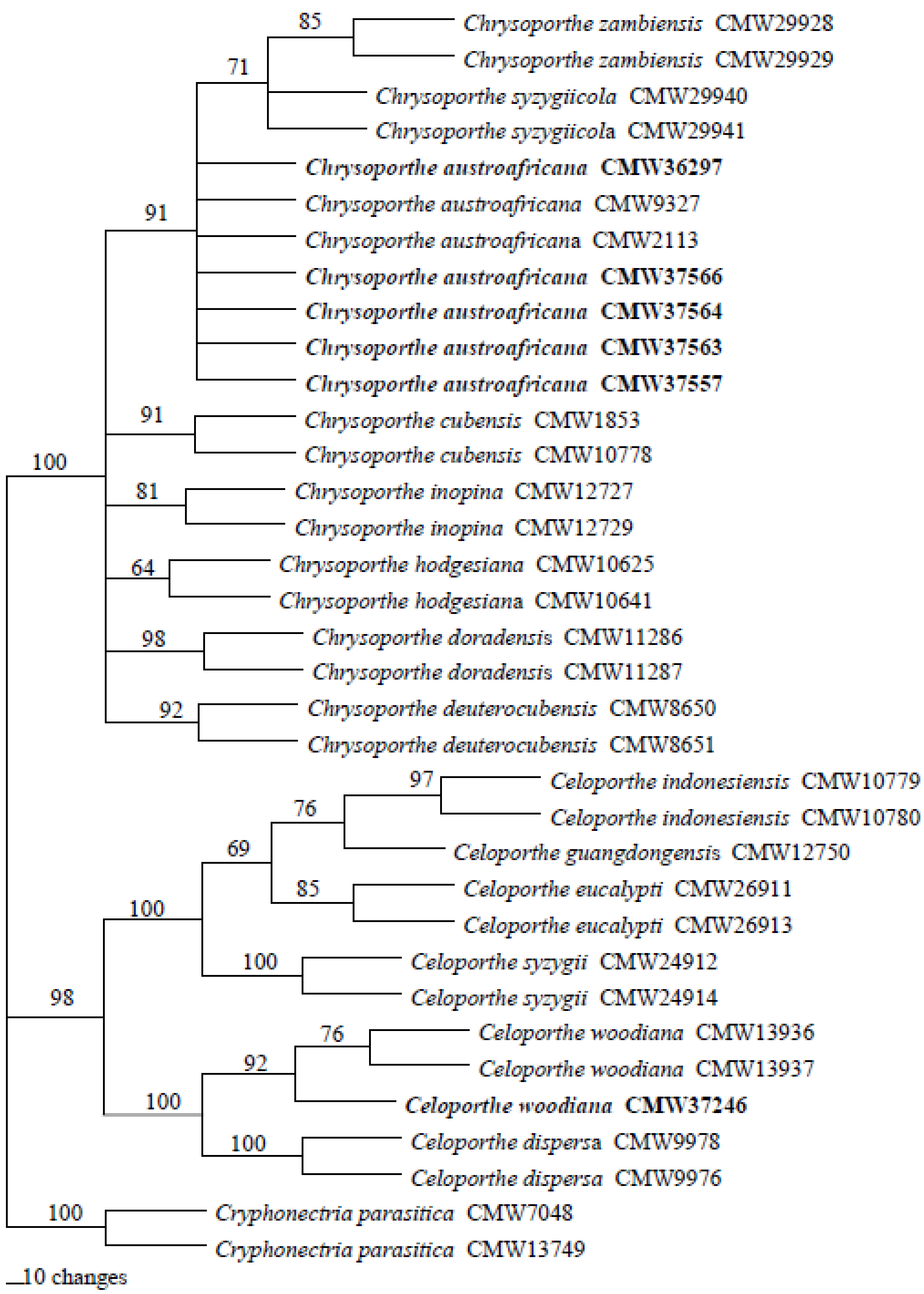

Figure 1. Phylogram of fungi in the Cryphonectriaceae indicating the phylogenetic positions of the fungal pathogen *C. austroafricana* and *Cel. woodiana* occurring on native Myrtales in Mozambique. Most parsimonious tree obtained from heuristic search of the combined ß-tubulin genes and Internal Transcribed Spacer (ITS) regions of the rDNA sequence data (TL

= 316, CI = 0.854, RI = 0.968, RC = 0.827, HI =0.146). Bootstrap confidence levels (1000 replicates) are indicated above the internodes. The tree is rooted to the out group taxa of *Cryphonectria parasitica*. Isolates obtained in this study are in bold. Only six isolates were chosen for representation in the trees. These were selected to represent each of the hosts and geographic areas from which the endophytic Chryphonectriaceae in this study originated.