CRYPHONECTRIACEAE FROM NATIVE MYRTALES IN LA RÉUNION AND SOUTH AFRICA

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by

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DECLARATION

I, Daniel Babangida Ali declare that the dissertation, which I hereby submit for the degree of *Master of Science* Microbiology at the University of Pretoria, is my own independent work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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PREFACE

The Cryphonectriaceae is a highly diverse group of fungi that are mainly parasites and endophytes with a few saprophytic species. *Chrysoporthe* and allied taxa are devastating tree pathogens especially of Myrtales (Myrtaceae and Melastomataceae). Every new region particularly tropical and sub-tropical areas of the world where trees in the Myrtales are examined for fungi in the Cryphonectriaceae seem to reveal previously unknown species of these fungi. Thus, description of new species is aided by molecular data using DNA sequence comparison combined with morphological characteristics.

The first chapter of this dissertation deals with a review of the major topics related to the Cryphonectriaceae. These include their taxonomic diversity, origin, identification tools, host jumps and shifts. It also summarizes the general approaches to working with fungi in the Cryphonectriaceae such as techniques for isolation and preservation. The Cryphonectriaceae represent a hugely diverse group of fungi with several new species only being discovered recently. Many areas where the hosts of these fungi grow are relatively under-explored and several species have yet to be adequately studied.

In the second chapter of this dissertation, a new genus and species *Myrtonectria myrtacearum* (prov. nom.) and two species of *Celoporthe* including *C. borbonica* (prov. nom.) and *C. tibouchinae* (prov. nom.) were described from native Myrtales in La Réunion and South Africa. Description of the new taxa was based on multi-gene sequence comparisons and morphological characteristics. Pathogenicity trials showed that the new taxa were mildly pathogenic to *Eucalyptus* (TAG 5) clone after 6 weeks of greenhouse inoculation trials.

CHAPTER 1

THE CRYPHONECTRIACEAE AND THEIR RISE AS THREATS TO FORESTS

Abstract

The Cryphonectriaceae, particularly *Chrysoporthe* and allied taxa, are important canker pathogens of Myrtales (Myrtaceae and Melastomataceae). These pathogens which occur in the tropical and sub-tropical parts of the world have been shown to cross-infect hosts especially from native trees to *Eucalyptus* grown plantations. Recent studies have shown that increased discovery of new species through molecular tools including those residing in asymptomatic plants as endophytes poses a serious threat to commercial *Eucalyptus* plantations. Thus, movement of plant material to *Eucalyptus* grown areas will threaten the expansion of these economic trees.

1.0. INTRODUCTION

The Cryphonectriaceae (Diaporthales, Ascomycota) is a highly diverse assemblage of fungi consisting of 23 genera and 74 species described from an estinmated 100 tree species in more than 14 plant families worldwide (Nakabonge *et al*. 2006a; Gryzenhout *et al*. 2006c; Gryzenhout *et al*. 2009; Cheewangkoon *et al*. 2009; Begoude *et al*. 2010; Vemeulen *et al*. 2013, Crous *et al*. 2012, 2015; Chen *et al*. 2012, 2013a, 2013b, 2016, 2017). The family accommodates fungi previously classified in the *Cryphonectria-Endothia* complex (Castlebury *et al*. 2002), and includes virulent pathogens, facultative parasites, endophytes and saprophytes (Hoegger *et al*. 2002; Gryzenhout *et al*. 2009; Mausse-Sitoe *et al*. 2016).

Members of the Cryphonectriaceae are distinguished by their orange stromatic tissues that turn purple in potassium hydroxide (KOH), and yellow in lactic acid (Gryzenhout *et al*. 2006c). This is because of the presence of pigments in their stromatic tissues or cultures (Rossman *et al*. 2007). Although Cryphonectriaceae and Nectriaceae (Hypocreales) have similar colour reactions in KOH and lactic acid (Rossman *et al*. 1999), colour can differentiate these taxa from other families or genera in the Diaporthales.

The most widely studied and recognized of the taxa in the Cryphonectriaceae are *Cryphonectria parasitica*, the chestnut blight fungus (Anagnostakis 1987; Dutech *et al*. 2012), *Chrysoporthe austroafricana* and *Chr. cubensis*, the *Eucalyptus* canker pathogens. *Cryphonectria parasitica* destroyed most of the American chestnut trees (*Castanea dentata*) in North America (USA) (Anagnostakis 1987; Dutech *et al*. 2012) and also caused substantial losses to these trees in Europe (Gurer *et al*. 2001; Dutech *et al.* 2010; Zamora *et al*. 2012; Hunter *et al.* 2013; Peters *et al*. 2014). In contrast, *Chr. austroafricana, Chr. cubensis* and allied taxa occur in the southern hemisphere where they cause stem canker disease on several tree genera in the Myrtales, most notably on plantation grown *Eucalyptus* species (Wingfield 2003). These fungi occur mostly in tropical and sub-tropical areas with high annual temperature and rainfall that favour infection and establishment of disease (Sharma *et al*. 1985; Hodges *et al*. 1986; Gryzenhout *et al*. 2009). These include South America (Boerboom and Maas, 1970), Central and North America (Hodges *et al*. 1979) and Asia (Sharma *et al*. 1985).

Over the past two decades, the number of Cryphonectriaceae genera and species known to science has increased exponentially. This can be attributed to increased numbers of reports of diseases caused by these fungi and changes in taxonomic tools used to define fungal species. The number of genera and species obtained from plants in the Myrtales (Combretaceae, Lythraceae, Myrtaceae and Melastomataceae) especially has more than doubled.

Every new region where trees in the Myrtales are examined for fungi in the Cryphonectriaceae seem to reveal previously unknown species of these fungi (Chungu *et al*. 2010; Begoude *et al*. 2010; Chen *et al*., 2011; Crane & Burgess 2013; Chen *et al*. 2013; Chen *et al*. 2016, 2017). This suggests that the diversity of species in the Cryphonectriaceae on these and other hosts remains under-studied in many regions of the world. This increased knowledge of their species diversity will help determine their ecological impacts, wide host ranges, and capacity to cross infect trees.

In this chapter, the diversity and taxonomy of the Cryphonectriaceae and their major impacts as tree pathogens in *Eucalyptus* plantations from the tropics and southern hemisphere is reviewed. Furthermore, the review explains host jumps/shifts that have occurred by these taxa, as well as providing insight into the different approaches used in working with fungi in the Cryphonectriaceae. These include methods of isolation, storage, identification and their existence as endophytes that constitute a potential threat to forest ecosystems when they are introduced into new environments.

2.0. CANKER DISEASE CAUSED BY CRYPHONECTRIACEAE SPECIES

Well known species of *Chrysoporthe* including *Chr. austroafricana, Chr. cubensis* and *Chr. deuterocubensis* are wound-infecting pathogens that cause Chrysoporthe canker disease (Wingfield 2003; Gryzenhout *et al*. 2009). Wounds are formed naturally by wind or insect damage. To test the pathogenicity of species of Cryphonectriaceae, wounds can be artificially induced on the stems and small amounts of mycelial inoculum, conidia, ascospores or spore suspension applied to the surface of the wounds to initiate infection (Boerboom and Maas 1970; Roane *et al*. 1974; Old *et al*. 1986; Old and Kobayashi 1988;

Yuan and Mohammed 1998; 2000; Gryzenhout *et al.* 2003; van Heerden *et al*. 2005). In *C. parasitica*, most infections occur where spores germinate on, and penetrate the bark through insect-induced wounds. As a result, cankers formed make the host water stressed and predispose it to infection (Anogstaskis *et al*. 1987; Hunter *et al.* 2013).

For many decades, Chrysoporthe canker has impacted susceptible trees in plantations of *Eucalyptus* in the tropics (Gibson 1981; Alfenas *et al*. 1983; Gryzenhout *et al*. 2004; van Heerden *et al*. 2005). The canker is characterized by orange or yellow fruiting structures formed on the dead bark (Gryzenhout *et al*. 2004). In severe cases, the cambium of stems are severely damaged and stems girdled, resulting in death of entire trees (Conradie *et al*. 1990; Wingfield 2003).

Chrysoporthe cubensis can cause severe cankers in areas of high rainfall and temperature (Sharma *et al*. 1985; Van Heerden *et al*. 2002). *Holocryphia eucalypti*, a *Eucalyptus* pathogen in Australia and South Africa, can cause mild or lethal cankers of trees that are grown in stressful conditions, such as drought, which leads to increased susceptibility of host trees (Old *et al*. 1986; Old *et al*. 1990; van der Westhuizen *et al.* 1993; Wardlaw 1999).

Disease severity and symptoms caused by well-known species of *Chrysoporthe* show considerable variation. For instance, *Chr. austroafricana* and *Chr. cubensis* are associated with various symptoms following infection of their hosts (Roane *et al*. 1986; Gryzenhout*et al*. 2004). *Chrysoporthe austroafricana* is the more aggressive of the two as it colonizes the cambium and girdles trees, rapidly resulting in death of young trees (Wingfield 2003). In contrast, *Chr. cubensis* causes cankers that extend to the trunks and infect more deeply resulting in more permanent cankers (Roux *et al*. 2003; Rodas *et al*. 2005). Both pathogens can cause cankers at the bases of trees and higher up on tree trunks (Sharma *et al.* 1985; Wingfield 2003; Nakabonge *et al*. 2006a).

3.0. TAXONOMY OF THE CRYPHONECTRIACEAE

3.1. Major taxonomic revisions in Cryphonectriaceae

The taxonomy of the Cryphonectriaceae has been a source of confusion since the description of two well-known genera, *Cryphonectria* and *Endothia* (Barr 1978). This confusion arose because early classification was based on morphological characteristics that overlapped among species and even genera. For example, *Cryphonectria* and *Endothia* were thought to be closely related members of the Diaporthales as they both possess orange fruiting structures and shared an anamorph genus, *Endothiella* (Kobayashi 1970; Roane *et al*. 1986). Furthermore, the conidia of *Endothia* and *Cryphonectria* are similar, and stromatal morphology of *Cryphonectria* species can be superficial and sometimes strongly developed similar to those of *Endothia*. However, Barr (1978) separated *Cryphonectria* from *Endothia* because it has fusoid to ellipsoid, one-septate ascospores, while *Endothia* has cylindrical to allantoid aseptate ascospores. Evidence from phylogenetic analyses conclusively showed that *Cryphonectria* and *Endothia* are paraphyletic, thus supporting their classification into two distinct lineages (Venter 2002; Myburg *et al*. 2004b).

The use of multigene phylogenies based on DNA sequence data, combined with morphological characteristics has provided considerable insight into the taxonomy of the Cryphonectriaceae (Gryzenhout *et al*. 2006; Gryzenhout *et al*. 2004; Myburg *et al*. 2004a; Myburg *et al*. 2002b;.Chen *et al*. 2013). Consequently, several species of fungi were transferred from genera in which they were originally described, into new genera and species. This led to systematic revisions in these groups based on a phylogenetic species concept (Table 2).

4.0. SPECIES DELINEATION IN THE CRYPHONECTRIACEAE

A species concept is fundamentally important in studies of evolution, ecology, phylogeny and conservation biology (Taylor *et al*. 2000; Cai *et al*. 2011). Traditional tools for defining fungal species in the past relied on isolation and culturing of environmental samples, and laboratory identification by morphology and biochemical tests (Atkins and Clark 2004; De Beer *et al*. 2016). These tools remain important as they show some degree of intraspecific plasticity (Parnmen *et al*. 2012). Morphological and biochemical methods have several limitations because they are time-consuming, laborious, and require extensive knowledge of classical taxonomy. These limitations were overcome by various molecular approaches such as molecular barcoding and phylogenetic species recognition that are more accurate and

reliable for detection, identification and delimitation of species (Schaad and Frederick 2002; Capote *et al*. 2003; McCartney *et al*. 2003; Schoch *et al*. 2012).

4.1. Morphological species concept

Morphological species recognition (MSR) was widely employed as a primary tool in fungal taxonomy (Taylor *et al*. 2000). Under this concept, new species were described based on their distinct morphological characteristics. However, one major problem of MSR is its inability to distinguish between closely related species and cryptic species, which are often morphologically identical, and for which morphology is not a reliable tool for estimating true species diversity. Morphological characters are used to distinguish genera of the Cryphonectriaceae, and species are distinguished with a combined morphological and molecular approach.

4.1.1. Morphological characteristics used for describing species

The morphological characteristics common to species in the Cryphonectriaceae are typical of other taxa in the Diaporthales, and constitute the *Diaporthe*-type centrum. These include perithecia with long necks found in the pseudostromata, absence of paraphyses between asci, and asci that possess thick walls that are either evanescent or intact (Castlebury *et al*. 2002). Members of the Cryphonectriaceae are stromatic, having spore bearing structures borne inside stromata consisting of masses of vegetative hyphae. The colour, type and extent of development of stromatic tissues, colour and length of perithecial necks as well as colour and shape of conidiomata are important morphological characteristics used for placement of members into different genera (Gryzenhout *et al*. 2009). For species delineation, spore shapes and sizes are important (Micales and Stipes 1987).

Species of Cryphonectriaceae are generally morphologically similar. For example, species of *Celoporthe* and *Chrysoporthe*, both important canker pathogens, are morphologically similar because they have black, superficial conidiomata that are similar in shape (Nakabonge *et al*. 2006). The two genera have ascomata that are similar in colour, however, the perithecial necks of *Celoporthe* are usually shorter and not always fuscous black. Both possess single septate ascospores and in some cases, the fruiting structures of *Celoporthe* occur together with those of *Chrysoporthe* on a piece of bark, and can be mistaken for

Chrysoporthe during isolation (Nakabonge *et al.* 2006). *Chrysoporthe* species also have a similar morphology to species of *Holocryphia*. The teleomorphs of species in both genera possess orange stromatal tissues (Venter *et al.* 2002; Gryzenhout *et al*. 2004; Gryzenhout *et al.* 2006a), and share common hosts and geographical distributions (Old *et al.* 1986; Davison and Coates 1991; Van der Westhuizen *et al*. 1993).

Genera in the Cryphonectriaceae differ more in their morphological features than species. For instance, *Holocryphia* and *Chrysoporthe* can be distinguished by their conidiomata and ascospores. *Chrysoporthe* has superficial, fuscous black, pyriform to orange conidiomata with attenuated necks and ascospores that are septate (Gryzenhout *et al*. 2004; Myburg *et al*. 2004), whereas conidiomata of *Holocryphia* are semi-immersed, orange to globose without necks and ascospores are aseptate (Gryzenhout *et al*. 2006e, Myburg *et al.* 2004a; Venter *et al.* 2002). The separation of these groups is also well supported in phylogenetic analyses (Gryzenhout *et al*. 2006c).

Cryphonectria and *Chrysoporthe* have differences in morphology particularly in their asexual states (anamorph), stromatal structures and septation of ascospores (Myburg *et al*. 1999; Myburg 2002b; Venter *et al*. 2001). *Cryphonectria* includes species that possess semi-immersed stromata, orange and pulvinate conidiomata with necks extending into short papillae on the stromatal surface; their ascospores are hyaline, ellipsoid to fusoid with one median septum (Gryzenhout *et al*. 2006d; Myburg *et al*. 2004b). In contrast, species of *Chrysoporthe* and allied genera possess superficial, blackened conidiomata, limited ascostromatal tissues, with blackened perithecial necks that protrude from the orange stromatal surface; their ascospores are hyaline, fusoid to oval, with one septum at different positions but mostly central (Gryzenhout *et al*. 2004).

4.1.2. Pleomorphic structures and the *Melbourne Code*

4.1.3. Pleomorphic structures in Cryphonectriaceae

The occurrence of two morphs (teleomorph and anamorph) in the life cycle of fungi in the Cryphonectriaceae is well known. These differences that occurred between the two morphs formed the basis for differentiating and describing new species (Myburg *et al*. 2004b; Gryzenhout *et al*. 2005d; Gryzenhout *et al.* 2006a; Gryzenhout *et al*. 2006b). Anamorphic

structures were prevalent among species in the Cryphonectriaceae, and Gryzenhout *et al.* (2009) suggested this stage should be adopted to describe species in these genera, particularly in *Chrysoporthe*.

4.1.4. *Melbourne Code* and its implications on naming of pleomorphic fungi

The anamorphic or asexual stage, and the teleomorphic or sexual stages of a fungus were treated as two different taxa under the first international rules for botanical nomenclature in 1867. This was termed dual nomenclature of fungi (Pirozynski and Weresub 1979; Taylor 2011) and was one of the most contentious topics among mycologists since the mid-19th century.

With the impact of molecular systematics it became obvious that fungi with no known teleomorph stage could be placed in genera typified by species with known sexual stages (Reynolds 1991; Shenoy *et al*. 2007;Taylor 2011b). This created polyphyletic groups of genera, and proposals to abandon the dual nomenclatural system were submitted for consideration (Hawksworth *et al*. 2009). The proposals were debated at several International Mycological Congresses, and on other platforms that emphasized the need to reconsider revision of the rules that govern dual nomenclature (Norvell *et al.* 2010; Redhead 2010; Wingfield 2011) .

One of the major concerns was non-compliance to the rules by scientists, due to different ways that molecular phylogenetic analyses might be interpreted (Seifert and Rossman 2010). Critical debates to provide one name for one fungal species as opposed to the system of dual nomenclature (Samuels *et al*. 2009) culminated in the Amsterdam Declaration published in 2011. At the 18th Botanical Congress held in Melbourne in July 2011, proposals were considered and adopted to modify the Vienna edition of the International Code of Botanical Nomenclature (ICBN) (McNeill *et al*. 2006; Mcneill and Turland 2011) and the discontinuation of dual nomenclature for pleomorphic fungi.

Consequently, a revised Code – the International Code of Nomenclature for algae, fungi and plants (ICN)–also known as the Melbourne Code (McNeill *et al.* 2012), as well as changes relating to fungal nomenclature in Article 59, were adopted. Thus, the principle of ‘‘One Fungus, One Name’’ (1F, 1N) for anamorphic fungal species was approved and included in

the new *Code* (Hawksworth *et al*. 2011b; Norvell 2011). The Melbourne Code which came to effect from January 2013 ended the use of separate generic names for anamorphs and teleomorphs. This implies that all legitimate names that were proposed for a fungal species, irrespective of what stage they are typified by, become part of a valid and accepted name for that species (Hawksworth 2011a).

4.2. Molecular methods

An approach to classification that resolved the limitations of MSR was the use of DNA sequence data for molecular barcoding and phylogenetic species recognition. The internal transcribed spacer (ITS1 and ITS2) regions, including the conserved 5.8S gene of the ribosomal RNA operon, was used to facilitate DNA based species recognition following its acceptance as a universal barcode locus for fungi (White *et al*. 1990; Schoch *et al*. 2012). *ITS* was selected as a molecular barcode for several reasons, including its high copy number presence in genomic DNA, its intraspecific conservation and interspecific differences, which allowed for amplification and species identification (Schoch *et al*. 2012).

4.2.1. Single genes in fungal identification

The Polymerase Chain Reaction (PCR) enabled specific gene regions of a genome to be amplified and sequenced using specific primers (White *et al*. 1990; Mullis *et al.* 1994; Glass and Donaldson 1995; Carbone and Kohn 1999). Following this development, Myburg *et al*. (1999) performed the first molecular work on Cryphonectriaceae using the analyses of PCR-amplified *ITS* to resolve taxonomic complexity between *Endothia* and *Cryphonectria*. Subsequently, other gene regions such as the small subunit (*SSU*), large subunit (*LSU*) of ribosomal DNA, and protein-coding genes such as actin, beta tubulin (*βt1 and βt2*), calmodulin, histone (*H3*) and translation elongation factor (*TEF-1α*), have also been used to delimit species in the Cryphonectriaceae (Table 3). These genes have been combined in phylogenetic analyses to differentiate among cryptic species.

4.2.2. MLST and GCPSR as tools for delineating cryptic species

Cryptic species (sibling species) refer to distinct species that are morphologically similar. Cryptic species complexes, particularly in fungi, occur frequently throughout the tree of life

and exist among single morphological or biological species with a cosmopolitan distribution that are often geographically separated (Dettman *et al*. 2003; Bickford *et al*. 2007). This is because fungi exhibit simple morphologies compared to macro-organisms, which makes it difficult to distinguish between recently diverged species or sibling species (Taylor *et al*. 2006). A number of cryptic fungal species have been named and placed into various taxonomic groups based on comparison of morphological characteristics. Thus, separation of such species within cryptic species complexes have been made possible by phylogenetic inference that grouped morphologically similar taxa into phylogenetic species (Taylor *et al.* 2000).

Although ITS was chosen as the formal fungal barcode for species identification, it sometimes fails to differentiate closely related phylogenetic species. This led to the advent of an empirical method, the genealogical concordance phylogenetic species recognition concept (GCPSR), for recognizing and distinguishing closely related isolates, cryptic species, and to infer evolutionary relationships between them (Dettman *et al*. 2003; Myburg *et al*. 2004a, van der Merwe *et al*. 2009; Vermeulen *et al*. 2013; Stergiopoulos and Gordon 2014). GCPSR involves sequencing of multiple gene regions across the genome, coined multi-locus sequence typing (MLST) (Taylor and Fisher 2003), that can be applied for genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al.* 2000; Stergiopoulos and Gordon 2014). GCPSR compares multiple gene regions in phylogenetic analyses and incongruent nodes are identified as the point of genetic isolation and species limitation (Taylor *et al.* 2000).

GCPSR has separated cryptic species, thus arriving at more precise classifications for many fungal genera (Hawksworth 2004). It has also been used to unravel complexes of cryptic species in the Cryphonectriaceae. For example, using this method, *Chr. cubensis* and *Chr. deuterocubensis* were separated into distinct species (van der Merwe *et al.* 2009). Similarly, two previously unknown cryptic species in *Celoporthe*, *C. woodiana* and *C. fontana*, were delineated based on GCPSR (Vermeulen *et al*. 2013).

5.0. ORIGIN AND MOVEMENT OF SPECIES IN THE CRYPHONECTRIACEAE

The centre of origin for a plant pathogen can be tested by population studies that seek the area with the highest genetic diversity (Stukenbrock and McDonald 2008; Linde and McDonald 2009). For example, one hypothesis on the origin of the chestnut blight pathogen, *C. parasitica*, is that the fungus originated from Japan and China. This hypothesis was proposed when low genetic diversity was observed among the isolates in Europe compared to higher diversity in the USA (Anagnostakis *et al*. 1986; Liu *et al*. 1996; Robin and Heiniger 2001), and very high diversity among isolates from China and Japan (Liu and Milgroom 2007). This fungus, that ravaged native American trees, was perhaps introduced into eastern America in the late 1800s, early 1900s with seedlings from Asia (Jaynes and DePalma 1984; Anagnostakis 1987).

There are two main hypotheses regarding the origin of *Chrysoporthe cubensis*. The first hypothesis was that *Chr. cubensis* originated from the Molucca Islands in Indonesia, since the fungus occurred on indigenous clove trees (*S. aromaticum*). The fungus was later moved to new areas, probably through the spice trade and infected *Eucalyptus* plants (Hodges *et al*. 1986). The claim that *Chr. cubensis* originated from Indonesia was not supported in separate studies by Van Heerden *et al*. (1997) and van Zyl *et al*. (1998), using Vegetative Compatibility Groups (VCGs). They found high genetic diversity respectively, within Venezuelan and Brazilian populations of the pathogen.

The discovery of the fungus on native trees in Colombia and Mexico has provided evidence for a second hypothesis, namely of a South American origin for this fungus (Rodas *et al*. 2005; Gryzenhout *et al*. 2006). This hypothesis is supported by genetic studies which showed that the fungus in African countries where it is also known, such as Cameroon, Democratic Republic of Congo, Republic of Congo and Ghana, has a low genetic diversity (Myburg *et al*. 1999, 2002b; Myburg *et al*. 2003; Roux and Apetorgbor 2010; Roux *et al*. 2003). (Myburg *et al*. 2002b; Roux *et al*. 2003; Gryzenhout *et al*. 2004; Nakabonge *et al*. 2006; Nakabonge *et al*. 2007). *Chrysoporthe cubensis* could not have originated from Africa given the fact it has never been reported on a native host on this continent.

Chrysoporthe deuterocubensis, previously confused with *Chr. cubensis* and *Chr. austroafricana*, is thought to have originated from South-eastern Asia and later introduced into several countries in eastern Africa, namely Kenya, Malawi, Mozambique and Zanzibar in Tanzania (van Heerden and Wingfield 2001; Myburg *et al*. 2003; Gryzenhout *et al*. 2004;

Nakabonge *et al*. 2006b; Nakabonge *et al*. 2007; van der Merwe *et al*. 2009). This claim regarding the origin of *Chr. deuterocubensis* is further supported by the presence of the pathogen on native Melastomataceae in Asia (Myburg *et al*. 2003; Gryzenhout *et al*. 2009).

The origin of *Chrysoporthe austroafricana* was unknown when it was first discovered in South Africa (Van Heerden and Wingfield 2001). An origin on this continent was not proposed because the fungus was only found on non-native *Eucalyptus* and was first treated as *Cryphonectria cubensis* (Conradie *et al*. 1990; Wingfield *et al*. 1989). Low genetic diversity in the populations of *Chr. austroafricana* was observed from non-native *Eucalyptus* spp. using vegetative compatibility groups (VCG's) (Van Heerden and Wingfield 2001). However, strong evidence later emerged for an African origin of *Chr. austroafricana* when it was found widely on native *Syzygium cordatum* and *S. guineense* in eastern and Southern Africa (Heath *et al*. 2006; Nakabonge *et al.* 2006b; Vermeulen *et al*. 2011; Mausse-Sitoe *et al*. 2017).

The discovery of *Chr. austroafricana* on native plants in Southern Africa was in contrast to earlier assertions that the fungus only infected non-native species of *Eucalyptus* and *Tibouchina granulosa* in South Africa (Conradie *et al*. 1990; Myburg *et al*. 2002a). Furthermore, absence of the fungus from other continents of the world supported its African origin. Equally convincing was the fact that *S. cordatum* showed more tolerance to infection by *Chr. austroafricana* than non-native *Eucalyptus* clones during pathogenicity trials (Heath *et al*. 2006). The tolerance of native *S. cordatum* to infection by the fungus supported a co-evolution hypothesis, where infection by a pathogen and resistance in a host, aside from other interferences, are at equilibrium in their native environment (Gilbert 2002).

Chrysoporthe austroafricana, having co-evolved with native trees in southern Africa has undergone host jumps and host shifts to infect non-native, naïve *Eucalyptus* and *Tibouchina granulosa* trees (Heath *et al*. 2006, Slippers *et al*. 2005). However, sufficient evidence was not established in the studies of Vermeulen *et al*. (2013a) about the movement of this pathogen between African countries. However, recent studies by Mkabili *et al*. 2017 (M. Sc. thesis) has further supported the southern African origin of *Chr. austroafricana* because highest diversity for the fungus was recorded in Mozambique indicating that it might have originated from this country.

Chrysoporthe doradensis is thought to be native to Ecuador because it is only known from *Eucalyptus* in that country (Gryzenhout *et al*. 2005b). A hypothesis follows that the fungus might have originated on native species of Myrtaceae in that region. Given that other species of *Chrysoporthe* in South and Central America also occur on native Melastomataceae, such as species of *Tibouchina*, it is probable that the fungus originated from one or more of these native Melastomataceae and Myrtaceae and underwent a host shift to *Eucalyptus* (Gryzenhout *et al.* 2004; Wingfield *et al*. 2002; Rodas *et al*. 2005).

Celoporthe species occur widely spread on their hosts in Africa and Asia, but the origin of these fungi has not been investigated. The isolates from Asia and Africa form two monophyletic groups that correspond to their regions of first detection (Vermeulen *et al*. 2013). Currently, three species occur in China and one species is known from Indonesia. Few studies have been conducted on *Celoporthe* in the latter area (Chen *et al.* 2011). All hosts that are infected by *Celoporthe* species in Asia are non-native to the areas. Conversely, surveys of Cryphonectriaceae in Africa have been conducted in several different countries including South Africa, Kenya, Mozambique and Namibia (Nakabonge *et al*. 2006a;Vermeulen *et al*. 2011b). Recent surveys of Cryphonectriaceae in South Africa and Zambia by Vermeulen *et al*. (2013b) recorded three additional *Celoporthe* species, two of which occur on native trees. This discovery prompted the authors to hypothesize that *Celoporthe* may have originated on native trees in Southern Africa.

It was hypothesized that *Holocryphia eucalypti* possibly originated from eastern Australia due its occurrence on *Eucalyptus* that are native trees in that region (Heath *et al.* 2007). Nakabonge *et al.* (2008) studied populations of *H. eucalypti* from Australia and South Africa using microsatellite data. Their findings revealed that isolates from eastern Australia were genetically more diverse compared to those from South Africa, implying that the fungus probably originates from eastern Australia. The fact that it occurs widely on native *Eucalyptus* spp. in native areas of Australia supports this hypothesis.

In a recent study on the diversity of *Holycryphia capensis* from the Western Cape Province, South Africa, using microsatellite markers, there was very low genotypic diversity (Chen *et al*. 2016). This suggests that *H. capensis* was probably introduced into the Western Cape from a single, as yet unknown origin. The origin of two newly described species of *Holocryphia*, namely *H. gleniana* and *H. mzansi*, from *Meterosideros angustifolia* in South

Africa, remains unknown. They were collected from a single location and in low numbers in the Western Cape Province of the country. For substantial evidence of the origin of these pathogens, sampling of known and potential hosts must be intensified in their supposed areas of origin.

6.0. HOST ASSOCIATIONS: HOST JUMPS/SHIFTS AND RANGE EXPANSION

There are increasing cases of new associations between fungi and naïve tree/plant species, also in the Myrtales, and which result in serious tree diseases (Alfenas *et al*. 2005; Slippers and Wingfield 2007; Zaffarano *et al*. 2008; Gilbert and Parker 2010; Wingfield *et al*. 2010). Most fungal pathogens have diversified through processes such as host shifts and host jumps (Slippers *et al*. 2005). Host shifts or jumps are defined when the hosts involved are taxonomically similar but geographically unrelated (host shifts), or taxonomically unrelated (host jumps) either in the same family or order to their original host (Slippers *et al*. 2005; Stukenbrock and Mcdonald 2008; Schulze-Lefert and Panstruga 2011; Silva *et al*. 2012). Host jumps and host shifts are direct consequences of successful host range expansion (Gladieux *et al*. 2011; Lê Van *et al*. 2011). This process can lead to the emergence of novel pathogens and disease through ecological speciation with serious impact on naïve host species (Zaffarano *et al*. 2008; Giraud *et al*. 2010).

Host jumps and host shifts occur when susceptible trees are introduced to a new area with potential new pathogens, or when pathogens are moved to a new area, often through accidental introductions of virulent species associated with agriculture and forestry (Wingfield *et al*. 2015; Burgess and Wingfield 2017). For example, *Cryphonectria parasitica*, which destroyed *Castanea dentata* in the USA (Anagnostakis 1987; Dutech *et al*. 2012), was thought to have been introduced from Asia to the USA in the early 1900's via the movement of Asian Chestnut trees, *Castanea crenata*. At that time, *C. dentata* was the dominant species in many forests in the eastern USA, but by the mid-1950's the blight had killed nearly 4 billion of these trees in their native ranges (Russell 1987).

A classical example of a host shift from native species of Myrtaceae to introduced *Eucalyptus* species was observed for *Chr. austroafricana*, a devastating canker pathogen of *Eucalyptus* in countries in east and southern Africa (Wingfield *et al*. 1989; Conradie *et al*. 1990; Heath *et al*. 2006; Nakabonge *et al*. 2006). The pathogen is thought to have shifted

from native species of *Syzygium* to non-native ornamental and plantation trees such as *Eucalyptus* and *Tibouchina* species in South Africa (Heath *et al*. 2006; Nakabonge *et al*. 2006). Similarly, *Chr. cubensis*, a pathogen of *Eucalyptus* species in South America has undergone host jumps/shifts from native tree species in the Myrtaceae and Melastomataceae (Wingfield 2003; Seixas *et al*. 2004; Rodas *et al*. 2005; Barreto *et al*. 2006).

A pathogen can expand its host range when it has the capacity to colonise a naïve introduced host species (Gladieux *et al*. 2011). For example, *Holocryphia eucalypti*, previously found on *Eucalyptus*, *Corymbia* and *Tibouchina urvilleana* in Australia and South Africa (van der Westhuizen *et al*. 1993; Carnegie 2007; Heath *et al*. 2007; Nakabonge 2010), has shifted and expanded its host range to infect several species of *Eucalyptus* in New Zealand and Uganda (Roux and Nakabonge 2010; Gryzenhout *et al*. 2010). Pathogens have rapidly expanded their host and geographical ranges and this is a global concern that needs a concerted effort to reduce their devastating impact (Wingfield *et al*. 2001;Wingfield *et al*. 2015).

7.0. WORKING WITH CRYPHONECTRIACEAE

7.1. Field observation and sample collection

Species of Cryphonectriaceae occur on bark and wood of trees and shrubs in natural forests, while some are found on economic trees grown in plantations or in gardens as ornamentals. Diseases caused by species of Cryphonectriaceae exhibit various symptoms, thus correct identification of these symptoms as well as fruiting structures on hosts in the field is useful in observing their presence. This can be aided by a hand lens and colour tests. Once a symptom is noticed, diseased bark or branch sections are collected and conveyed to the laboratory for morphological study and fungal isolations. Fruiting structures of Cryphonectriaceae are easily confused with those of other micro fungi, thus a colour reaction test is necessary to ensure their presence on diseased samples (Castlebury *et al*. 2002).

7.2. Isolations

Isolations from pieces of bark bearing fruiting structures that resemble those of Cryphonectriaceae depend on whether the fruiting structures are dried or freshly collected. Isolation techniques have been outlined by Gryzenhout *et al*. (2009). A sporulation method where pieces of bark are incubated in moist-chambers is not recommended, as it encourages rapid growth of opportunistic fungi, such as species of *Penicillium* and *Trichoderma*, which contaminate cultures. Furthermore, material for future morphological studies must not be incubated in moist chambers to help avoid loss of asci and conidia, which are important features for identification. For endophytic isolations, asymptomatic stem branches (twigs) are collected and sporulation of fruiting structures on twigs can be done following the methods of Beier *et al.* (2014) and Mausse-Sitoe *et al*. (2016).

Single spore isolation is necessary to ensure purity of cultures, especially when isolates are to be compared in phylogenetic or population genetic studies. To make pure isolates, single spore or single hyphal tip transfers can be made from colonies following the method by Gryzenhout *et al.* (2009). In most cases, serial dilution is required in order to get suspensions with a low dilution of spores that can be separated on the agar surface; this is important when conidial masses contain a large number of spores (Gryzenhout *et al*. 2009).

7.3. Culture characteristics and inducement of fruiting structures

Fungi growing in pure culture have been used for characterization of species (Slippers *et al.* 2004b; Crous *et al.* 2007; Phillips *et al*. 2013). Growth in culture can be compared under the same conditions at different temperatures. This is important in order to determine the optimal temperature for growth and to assess colony morphology. Colours of colonies are compared using the standard colour charts of Rayner (1970). To assess the temperature at which species grow, five temperatures are selected at 5 ^{o}C internal usually from 10 to 35 ^{o}C. Five replicate plates per isolate are used and two measurements perpendicular to each other are taken for each plate. Generally, Cryphonectriaceae species grow fast and mycelia can cover a 90 mm diameter plate within 4–7 days at the optimum growth temperatures of 25–30 ^{o}C (Gryzenhout *et al*. 2009).

Colours of colonies are usually white and fluffy when young and turn to various colours with age. These colours range from oliveceous brown, chestnut brown, cinnamon, crimson, orange and luteous, and are important for distinguishing between species. For example,

Celoporthe dispersa produces cultures that are white with grey to chestnut-coloured patches (Nakabonge *et al*. 2006; Vermeulen *et al*. 2013). These characteristic colours distinguish it from species of *Chrysoporthe* that are white to cinnamon-colour with hazel patches (Grezenhout *et al*. 2004; Myburg *et al*. 2003). Some species are known to produce pigments in cultures. *Cryphonectria radicalis*, for example, releases a characteristic red pigment known as endothine red, which colours the medium on which it grows (Roane *et al*. 1986); *Cryphonectria naterciae* produces a reddish purple pigment on corn meal (Braganca *et al*. 2011).

Fruiting structures (anamorphs and teleomorphs) can be stimulated through artificial inoculations or they can be induced on media supplemented with branch or stem pieces of host plants to give an approximation of the natural morphology (Chen *et al*. 2016; Myburg *et al*. 2002b; Myburg *et al.* 2003). Anamorph structures are produced in cultures but most species in the Cryphonectriaceae do not sporulate after sub-culturing from primary cultures. Teleomorph structures are rarely produced on artificial media but these can be produced on old, dried out cultures (Walker *et al*. 1985; Chen *et al*. 2016). Fruiting structures can be induced artificially on wood of the original material. In this case, fresh branches or twigs are used after sterilizing with ethanol and the cut ends sealed with hot paraffin or wax (Hodges *et al*. 1986). These pieces of plant material are then inoculated with a disc of fungus mycelium after removal of the bark with a cork borer (between 3–5 mm diam.). Inoculated wounds are sealed with paraffin or masking tape and incubated in a moist chamber for a month or much longer.

Morphological structures induced on artificial MEA can differ from those growing in nature. This has been observed in *Chrysoporthe* spp. as well as in *Corticimorbus sinomyrti* (Myburg *et al*. 2002a; Chen *et al*. 2016). For example, Chen *et al*. (2016) observed some differences in the naturally occurring ascostromata and those induced on artificial media in *C. synomyrti*, where in the former, they were more deeply immersed in the host tissue, and perithecia formed in culture had wider necks and were longer than in the latter. However, sexual structures including colour and type of tissue for the ascostromata, shape, colour and size of perithecia, as well as shape and size of asci and ascospores produced in nature did not differ from those produced in culture. Fruiting structures produced in cultures are only useful in taxonomic studies after they show similarity with those from naturally infected plant tissues (Gryzenhout *et al*. 2009).

7.4. Storage of cultures and fruiting structures

The preservation and maintenance of plant pathogenic fungi in a viable state for a long period of time is important because isolates of these fungi serve as a standard reference for 1) identification of quarantine taxa, 2) for testing disease resistance, and 3) breeding programmes (Abd-Elsalam *et al.* 2010). Considering the use of molecular sequences in phylogenetic studies in the recent years, methods of long term preservation are becoming increasingly important to ensure fungal survival and to retain any valuable characteristics for future studies (Abd-Elsalam *et al*. 2010). The main purpose of culture preservation is maintenance of vigour and genetic characteristics of a pure culture.

Cultures of Cryphonectriaceae are stored long term using malt yeast agar (MYA, 20g/L malt extract, 15g/L agar, 2g/L yeast extract). Oatmeal agar can also be used. It is prepared by autoclaving 50-60g/L oats, and then solids are removed from the autoclave broth with cheese cloth or muslin bags. The extract is then added to 17g/L agar and poured into McCartney bottles and cultures can be grown on the agar slants. The surfaces of the bottles are mostly covered with sterilized mineral oil or in some cases; the tops are sealed with cigarette papers. Cultures can also be freeze dried on glass beads or small pieces of wood, or they can be stored at –70 ^{o}C (Gryzenhout *et al.* 2009).

Different storage methods must be used to keep the cultures viable. For example, species of *Chrysoporthe* cannot survive long in culture (Gryzenhout *et al*. 2009), thus regular transfer of cultures onto fresh media should be maintained, but original copies of the cultures must be kept. Sterilized water can be used to store pieces of mycelium-covered agar at the temperature of –70 ^{o}C. For *Chrysoporthe* spp., more than one storage method is recommended because they do not survive well in culture. Long term storage is done on malt yeast agar or oats agar. Cultures can be grown in bottles containing agar slants and stored at 5–8 ^{o}C. Mycelial surfaces can be covered with sterilized mineral oil.

7.5. Microscopic examinations of fruiting structures

Morphological characteristics are examined on canker samples containing both sexual and asexual fruiting structures, although sexual structures rarely occur in most species of the Cryphonectriaceae. Morphological characteristics of the fruiting structures are studied by

cutting the structures from the specimens under a dissecting microscope, boiled in water for 2 min and sectioned using the methods of Gryzenhout *et al.* (2009). The asci, ascospores, conidiophores, conidiogenous cells and conidia are observed after crushing fruiting structures on microscope slides in sterilized water or 85% lactic acid. For a description from a holotype specimen, 50 measurements are made for each morphological structure, while 25 measurements per character can be made for the remaining specimens. Characteristics of specimens are compared with previously published data on Cryphonectriaceae that are related to the genera or species being described. Evaluation of growth in culture is done following the methods of Chen *et al*. (2016). Colour descriptions are studied using the colour manual of Rayner (1970).

8.0. ENDOPHYTIC CRYPHONECTRIACEAE AS A THREAT TO FORESTS

Endophytic fungi remain in plant tissues without causing immediate disease symptoms (Petrini 1992; Stone *et al*. 2004). This is analogous to latent infection where a host is infected by a potential pathogen (an endophyte) without visible signs, only for macroscopic disease to appear when hosts are stressed, resulting in canker disease (Stanosz *et al*. 2001, Vannini *et al.* 1996; Cheplick 2007). Fungal endophytes have also been considered as parasites, weak pathogens, or saprophytes that inhabit plants without functioning until the host is stressed or senescent (Saikkonen *et al*. 2004).

Endophytes have several beneficial roles. They have been shown to enhance host resistance to pathogens (Arnold *et al*. 2003), improve drought tolerance (Hubbarb *et al*. 2012), enhance growth (Ren *et al*. 2011), defend against invertebrate herbivory (Vesterlund *et al*. 2011), or serve as bio-control agents (Arnold *et al.* 2003). They occur in all plant species, and a number of them have been reported in grasses and woody plants (Schardl *et al*. 2012; Marsberg *et al*. 2014, Beier *et al*. 2014).

Several fungal endophytes show specificity to host organs (Mostert *et al*. 2000; Moricca *et al*. 2012) and grow asymptomatically inside host tissues including seeds, roots, stems or leaves. For example, *C. parasitica* was found in the phellem of the coppice shoots of *Castanea sativa* but absent in the pith and the xylem (Bissegger and Sieber 1994).

The occurrence of virulent fungi as latent (endophytic) species has serious implications on their spread through movement of plant material and germplasm as they remain undetected in plant tissues. This was the case of *C. parasitica* that spread and established in Europe because it existed as endophyte in the nutshells of *C. sativa* that were transported into the Europe from USA (Jaynes and DePalma 1984; Hunter *et al.* 2013). The recent discovery of pathogenic species of Cryphonectriaceae as endophytes, for example, *Chr. austroafricana* (Mausse-Sitoe *et al*. 2016) clearly indicates that once this fungus is accidentally introduced to other parts of the world particularly Australia, where *Eucalyptus* and many Myrtales are native, it could result in a serious threat to these trees. This raises a serious concern as climate change and other related environmental factors continuously inflict stresses on plants thus becoming more vulnerable to infection by latent endophytes.

9.0. CONCLUSIONS

This review has provided a summary of the taxonomy and host associations in the Cryphonectriaceae. Taxa in this family are morphologically and ecologically diverse plant pathogens, and are an emergent threat to *Eucalyptus* and several species of Myrtales particularly in the tropical and subtropical parts of the world. Much has been learnt from the devastating impact of *C. parasitica* in the Northern Hemisphere where it nearly destroyed the entire population of American chestnuts from their natural range in the mid-1950's. The increasing threats posed by species in the Cryphonectriaceae have recently generated serious concern in the Southern Hemisphere due to the debilitating stem canker disease and dieback symptoms they cause with direct consequences on growth and expansion of *Eucalyptus* plantations in these regions.

In the last 100 years, substantial losses have been recorded in plantations in the tropical and subtropical parts of the world due to epidemics caused by the Cryphonectriaceae. An unprecedented increase in the movement of potentially dangerous species as a result of global plant trade, as well as climate change, will further increase their rate of introductions and spread in new areas where they may cause disease on *Eucalyptus*. The risks posed by these pathogens will require a global strategy in order to prevent or lessen their devastating impacts through disease monitoring and imposition of strict quarantine regulations on movement of plant material. The reliance on visual symptoms of disease in plant parts as part of the phytosanitary measures to prevent introduction of fungal plant pathogens has led

to unintentional introductions of several endophytic species, which remain undetected in plant tissues. Thus rapid and reliable diagnostic tools are required to detect these pathogens *in plantae*.

The aim of this review was to highlight topics related to the Cryphonectriaceae including their morphology, taxonomy, origin, host jumps and shifts, disease symptoms, species delineation as well as threats posed by virulent species including those that exist as endophytes. In the next chapter of this thesis, a new genus and three species in the Cryphonectriaceae are described from native Myrtales in La Réunion and South Africa, using molecular data and morphology.

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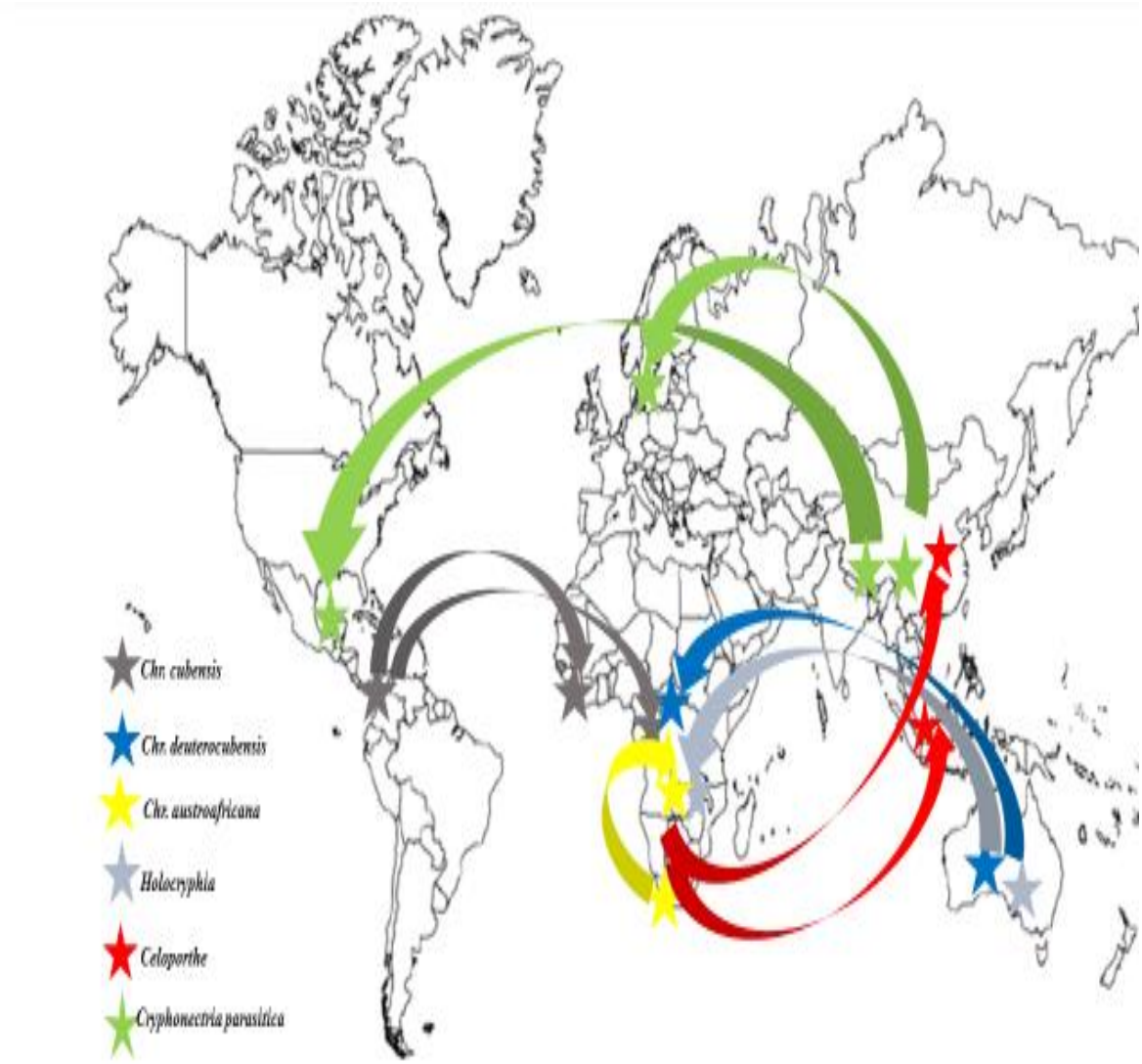

Fig. 1: Centres of origin and worldwide movement of species in the Cryphonectriaceae

Table 1: *Chrysoporthe* and allied taxa on *Eucalyptus* and other Myrtales

Species	Host	Area of occurrence	References
Aurapex penicillata Gryzenh. & M. J. Wingf.	*Miconia theaezans, T. urvilleana, T. lepidota*	Colombia	Gryzenhout *et al*. 2006
Aurifilum marmelostoma Begoude, Gryzenh. & Jol. Roux	*Terminalia ivorensis* and *T. mantaly*	Cameroon	Begoude *et al*. 2010
Celoporthe dispersa Nakab., Gryzenh., Jol. Roux & M.J. Wingf.	*Heteropyxis canescens*, *T. granulosa S. cordatum* and *S. legatii*	South Africa	Nakabonge *et al*. 2006 Vermeulen *et al*. 2011b
	S. guineense	Zambia	Vermeulen *et al*. 2011b
Celoporthe eucalypti S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou	*Eucalyptus,*	China	Chen *et al*. 2011
Celoporthe syzygii S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou	*Syzygium cumini*	China	Chen *et al*. 2011
Celoporthe guangdongensis S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou	*Syzygium aromaticum*	China	Chen *et al*. 2011
Celoporthe indonesiensis S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou	*Syzygium aromaticum*	Indonesia	Chen *et al*. 2011
Celoporthe fontana M. Verm., Gryzenh. & Jol. Roux	*Syzygium guineense*	Zambia	Vermeulen *et al*. 2013
Celoporthe woodiana M. Verm., Gryzenh. & Jol. Roux.	*Tibouchina granulosa*	South Africa	Vermeulen *et al*. 2013
Chrysoporthe austroafricana. Gryzenh. & M. J.Wingf	*Tibouchina granulosa*	Malawi, Mozambique	Nakabonge *et al.* 2006
	S. cordatum and *Tibouchina* spp.	South Africa	Myburg *et al*. 2003
	S. guineense	Namibia	Vermeulen *et al*. 2011a.
Chrysoporthe cubensis (Bruner) Gryzenh. & M. J. Wingf	*Tibouchina* spp	Australia	Pegg *et al.* 2010
Chrysoporthe cubensis	*Marlierea edulis*	Brazil	Barreto *et al*. 2006
	T. granulosa and S. aromaticum	Brazil	Hodges *et al*. 1986; Seixas *et al*. 2004
	Miconia spp.	Colombia	Rodas *et al.* 2005

Table 1: Continued

Species	Host	Area of occurrence	References
Chrysoporthe cubensis	*Eucalyptus* spp.	Brazil, Cameroon, Cuba, DR. Congo, Florida, Ghana, Kenya, Hawaii, Hong Kong, Indonesia, Malawi, Mozambique, Puerto Rico, Rep. of Congo, Surinam, Tanzania, Thailand, Trinidad, Vietnam, Western Samoa, Zambizar.	Boerboom and Maas 1970; Chen *et al*. 2010; Gibson 1981; Hodges 1980; Hodges *et al*. 1986; Nakabonge *et al.* 2006; Sharma *et al*. 1985; Roux *et al*. 2003; Roux and Apetorgbor 2010; Pegg *et al.* 2010).
Chrysoporthe deuterocubensis Gryzenh. & M. J. Wingf.	*Eucalyptus* spp. and *Syzygium* spp.	Australia, Southern China, Rep. of Congo	Chen *et al*. 2010; (Myburg *et al*. 2002); Hodges 1980; Hodges *et al*. 1986; Zhou *et al*. 2008; Roux *et al*. 2003; van der Merwe *et al*. 2009.
Chrysoporthe doradensis Gryzenh. & M.J. Wingf.	*Eucalyptus and T. urveleana*	Ecuador	Gryzenhout *et al*. 2005
Chrysoporthe hodgesiana (Gryzenh. & M.J. Wingf.) Chungu, Gryzenh. & M.J. Wingf.	*Miconia theaezans*	Colombia	Gryzenhout *et al*. 2004
Chrysoporthe inopina Gryzenh. & M. J. Wingf.	*Tibouchina lepidota*	Colombia	Gryzenhout *et al*. 2006
Chrysoporthe syzygiicola Chungu, Gryzenh. & Jol. Roux	*Syzygium guineense*	Zambia	Chungu *et al*. 2010b
Chrysoporthe zambiensis Chungu, Gryzenh. & Jol. Roux	*Eucalyptus grandis*	Zambia	Chungu *et al*. 2010b
Cryphonectria parasitica (Murill) Barr	*Eucalyptus* spp.	Japan	Old and Kobayashi 1988
Corticimorbus sinomyrti S.F. Chen, F.F. Liu & M.J. Wingf.	*Rhodomyrtus tomentosa*	China	Chen *et al.* 2016
Cryptometrion austuescens	*Eucalyptus* spp.	Indonesia	Gryzenhout *et al*. 2010
Diversimorbus metrosiderosis S.F. Chen & Jol. Roux	*Metrosideros angustifolia*	South Africa	Chen *et al*. 2013

Table 1: Continued

Species	Host	Area of occurrence	References
Holocryphia eucalypti (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf.	*Eucalyptus* spp./clones	Australia, China, Florida, India, Japan, New Zealand, South Africa, Swaziland; Uganda.	Kobayashi 1970; Walker and Murray 1985; Old *et al*. 1986; Barnard *et al*. 1987; Davison and Coates 1991; van der Westhuizen *et al*. 1993; Gryzenhout *et al*. 2003; 2006a; Heath *et al*. 2007; 2010b; Roux and Nakabonge 2010.
	Corymbia	Australia	Walker *et al*. 1985; Old *et al*. 1986.
Holocryphia mzansi S.F. Chen. & Jol. Roux	*Metrosideros angustifolia*	South Africa	Chen *et al*. 2013
Holocryphia capensis S.F. Chen. & Jol. Roux	*Metrosideros angustifolia*	South Africa	Chen *et al*. 2013
Holocryphia gleniana S.F. Chen. & Jol. Roux	*Metrosideros angustifolia*	South Africa	Chen *et al*. 2013
Immersiporthe knoxdaviesiana S.F.Chen., M.J. Wingf. & Jol. Roux	*Rapanea melanophloeos*	South Africa	Chen *et al*. 2013
Latruncelllus aurorae M. Verm., Gryzenh. & Jol. Roux	*Galphinia transvaalica*	Swaziland	Vermeulen *et al*. 2011b

Table 2: Taxonomic Revision in the Cryphonectriaceae

Current name	Year renamed	Previous name (s)	Year described	References
Cryphonectriaceae Gryzenh. & M.J. Wingf.	2006	*Diapothaceae*; *Valsaceae*	2002	Castlebury *et al*. 2002; Gryzenhout *et al*. 2006c)
Chrysoporthe cubensis (Bruner) Gryzenh. & M.J. Wingf.	2004	*Diaporthe cubensis* Bruner	1976	Bruner 1916; Gryzenhout *et al*. 2006c)
		Cryphonectria cubensis (Bruner) Hodges	1980	Hodges *et al*., 1986)
-		*Cryptodiaporthe* Roane	1986	Roane 1986a
Chrysoporthe hodgesiana (Gryzenh. & M.J. Wingf.) Chungu, Gryzenh. & M.J. Wingf.	2010	*Chrysoporthella hodgesiana* Gryzenh. & M.J. Wingf.	2004	Gryzenhout *et al*. 2006c; Chungu *et al*. 2010.
Cryphonectria parasitica	1972	*Endothia parasitica*	1917	Bruner 1916; Stipes *et al*. 1978.
Rostraureum tropicale Gryzenh. & M.J. Wingf.	2005	*Cryphonectria longirostris* (Earle) Micales & Stipes	1901	Gryzenhout *et al*. 2005a
Microthia havanensis (Bruner) Gryzenh. & M.J. Wingf.	2006	*Cryphonectria gyrosa* (Berk. & Broome) Sacc	1970	Kobayashi 1970; Hodges *et al*. 1986)
-		*Cryphonectria havanensis* (Bruner) M.E. Barr	1987	Barnard *et al*. 1987.
Chrysoporthe deuterocubensis Gryzenh. & M.J. Wingf.,	2009	*Chrysoporthe cubensis*	2004	Gryzenhout *et al*. 2004; van der Merwe *et al*. 2009.
Holocryphia eucalypti (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf	2006	*Endothia gyrosa*	1917	Shear *et al*. 1917; Roane *et al*. 1974; Appel and Stipes 1986; Gryzenhout *et al*. 2006a
		Cryphonectria gyrosa	1983	Sharma *et al*. 1985; Old *et al*. 2003
		Cryphonectria eucalypti M.Venter & M. J. Wingf	2002	Venter *et a*l. 2002

Table 3: **Important gene regions used in delineating species in the Cryphonectriaceae**

Group/species delineated	Gene region (s) used	Year	Author (s)
Celoporthe fontana & C. woodia	TEF-1α	2013	(Vermeulen *et al*. 2013)
Chrysoporthe cubensis	ITS	1999	(Myburg *et al*. 1999)
Chr. cubensis and allied species	*BT* and *Histone 3*	2002	(Myburg *et al*. 2002a)
Chr. austroafricana & Chr. cubensis	*BT* and *Histone H3*	2004	(Gryzenhout *et al*. 2004)
Diaporthales	LSU	2002	(Castlebury *et al*. 2002)
Cryphonectriaceae	LSU & ITS	2006	(Gryzenhout *et al*. 2006c)
Chrysoporthe deuterocubensis	BT, ACTIN and TEF-1α	2009	(van der Merwe *et al*. 2009)
Holocryphia capensis, H. gleniana and *H. mzansi*	ACT, BT and TEF	2013	(Chen *et al*. 2013a)

CHAPTER TWO

NOVEL TAXA OF CRYPHONECTRIACEAE FROM LA RÉUNION AND SOUTH AFRICA AND THEIR POTENTIAL THREAT TO SPECIES OF *EUCALYPTUS*

Abstract

The Cryphonectriaceae is a family of important fungi that infect bark and cause cankers ontrees of many species.Species of Cryphonectriaceae are reported to switch hosts through jumps or shifts, which includes examples of fungi, such as *Chrysoporthe austroafricana* and *C. cubensis*, now regarded as severe pathogens in plantations of *Eucalyptus*. In this study, fruiting structures resembling those of Cryphonectriaceae were collected and isolated from cankered, dying branches of *Syzygium cordatum* and root collars of drying *Heteropyxis natalensis* in New Agatha, Limpopo Province, South Africa. In addition, fungi isolated from bark samples of native *Tibouchina grandifolia* in La Réunion and deposited in the CMW culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, were also studied. A phylogenetic species concept was used to identify these species of Cryphonectriaceae using partial sequences of the Large Subunit and, Internal Transcribed Spacer regions of nuclear ribosomal DNA, and two regions of the b-tubulin (BT) gene. The results arising from the phylogenetic hypotheses revealed an undescribed genus of Cryphonectriaceae from South Africa, and two undescribed species of *Celoporthe* from La Réunion. These new taxa will be described as *Myrtonectria myrtacearum* gen. et sp. nov. (prov. nom.), *Celoporthe tibouchinae* sp. nov.(prov. nom.) and *Celoporthe borbonica* sp. nov.(prov. nom.) The three new taxa were found to be mildly pathogenic on *Eucalyptus* following an inoculation trial. This study reaffirms that latent or potential pathogens can be unintentionally moved between areas, and constitute a serious threat to ecosystem services provided by trees.

1.0. INTRODUCTION

The Cryphonectriaceae (Diaporthales, Ascomycota) accommodate fungi previously classified under the *Cryphonectria-Endothia* complex (Castlebury *et al*., 2002; Gryzenhout *et al*. 2006c), and include facultative parasites (Gryzenhout *et al*., 2009), endophytes (Beier *et al*., 2014; Mausse-Sitoe *et al*., 2016) and saprophytes (Hoegger *et al*., 2002; Myburg *et al*., 2004). This diverse fungal family has 23 genera and 74 species (Gryzenhout *et al*., 2005a; 2005b; Gryzenhout *et al*., 2010; Chen *et al*., 2016; Chen *et al*., 2017). Seven of these genera have been reported in Africa including *Aurifilum* (Begoude *et al*., 2010), *Celoporthe* (Nakabonge *et al*., 2006a), *Chrysoporthe* (Gryzenhout *et al*., 2004), *Diversimorbus* (Chen *et al*., 2013b), *Holocryphia* (Gryzenhout *et al*., 2006a), *Immersiporthe* (Chen *et al.*, 2013a) and *Latruncellus* (Vermeulen *et al*., 2011).

Some species of Cryphonectriaceae are regarded as high risk pathogens because they cause serious canker and die-back symptoms and have undergone host switches between families and genera (Slippers *et al*., 2005; Van der Merwe *et al.*, 2013; Wingfield *et al*., 2015; Burgess & Wingfield 2017). For example, *Chrysoporthe austroafricana* having co-evolved with native trees in southern Africa has undergone host switches to infect non-native, naïve *Eucalyptus* and *Tibouchina granulosa* trees (Myrtaceae, Myrtales) (Conradie *et al*. 1990; Myburg *et al*. 2002b; Heath *et al.*, 2006; Nakabonge *et al*., 2006b; Vermeulen *et al*., 2011). A second species of *Chrysoporthe*, *Chr. cubensis*, caused cankers on species of *Eucalyptus* after a host shift from native species of Melastomataceae in South America (Seixas *et al*., 2004; Rodas *et al*., 2005; Van der Merwe *et al.* 2013).

In Africa, species of Cryphonectriaceae infect several genera of the Myrtales including *Eucalyptus*, *Heteropyxis*, *Metrosideros*, *Syzygium* (Myrtaceae) (Myburg *et al*., 2002b; Nakabonge *et al*., 2006b; Chen *et al*., 2013b, Heath *et al*., 2006), *Tibouchina* (Melastomataceae) (Myburg *et al.*, 2002a) and *Terminalia* (Combretaceae) (Begoude *et al*., 2010). These plants are either non-native species planted as ornamentals and for forestry, such as *Tibouchina* spp., and *Eucalyptus* , or are native to South Africa, such as species of *Heteropyxis, Metrosideros, Syzygium* and *Terminalia* (Myburg *et al*., 2002b; Nakabonge *et al*., 2006b; Chen *et al*., 2013b). A genus of Cryphonectriaceae, *Diversimorbus*, has also been reported from *Rapanea* (Primulaceae, Ericales) in South Africa (Chen *et al*., 2013a).

Celoporthe was first described from *C. dispersa* on species of Melastomataceae and Myrtaceae in South Africa (Nakabonge *et al*., 2006a). *Celoporthe* differs from other genera in the Cryphonectriaceae by its ascostromata that consist of black, valsoid perithecia with short, attenuated perithecial necks, but is similar to *Chrysoporthe* in morphology by its superficial conidiomata that are orange to scarlet when young and fuscous-black when mature (Nakabonge *et al*., 2006a). The type species was found to be pathogenic on *Eucalyptus*, and other species of *Celoporthe* have since been described from *Eucalyptus*. These taxa, which cause stem cankers, are biosecurity risks.

There are six species of *Celoporthe*, including the type, associated with canker and die-back on branches and stems of native and introduced Myrtales. The type species is restricted to South Africa and has been found on native *S. cordatum*, *Heteropyxis canescens* and non-native *T. granulosa* (Nakabonge *et al*., 2006a). Vermeulen *et al*. (2013) described *C. fontana* and *C. woodiana*, which were not conspecific with *C. dispersa*, but occurred on the same hosts in South Africa and Zambia (Nakabonge *et al*., 2006b; Vermeulen *et al*., 2011, Vermeulen *et al*., 2013). A study by Chen *et al.* (2011) further described *C. eucalypti*, *C. syzygii* and *C. guangdongensis* from *Eucalyptus* and *S. aromaticum* in China, and *C. indonesiensis* from *S. aromaticum* in Indonesia. These taxa have been defined based on morphology and a phylogenetic species concepts (Taylor *et al*., 2000; Gryzenhout *et al*., 2009).

Several fungi with orange fruiting structures resembling species of Cryphonectriaceae and that caused girdle-cankers on species of Melastomataceae and Myrtaceae were found during disease surveys of native forests in La Réunion and South Africa. The aim of this study was to (i) characterize these fungi within the Cryphonectriaceae using morphology and a phylogenetic species concept, and (ii) determine their pathogenicity to *Eucalyptus*.

2.0. MATERIALS AND METHODS

2.1. *Sampling and isolation of fungi*

Isolates used in this study were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa,

as well as from two surveys of native Myrtales in Tzaneen, Limpopo Province (South Africa). Surveys of native Myrtales targeted disease symptoms such as stem or root collar cankers and die-back with orange or yellow fruiting structures on their surface. Sections of stem and root-bark were collected and transported in sterile brown paper bags to the laboratory for fungal isolation.

Spore masses within fruiting structures were exposed and transferred with sterile needles onto 2% Malt Extract Agar (MEA) with 100 mg/L streptomycin sulphate added to inhibit bacterial contamination. Plates were incubated at 25 °C for fungal growth. Pure cultures were obtained from the colonies by transferring single hyphal tips to MEA. Cultures were deposited in the Culture Collection (CMW) of FABI, University of Pretoria, South Africa, and representative cultures will be deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands (Table 1). Original bark specimens with fruiting structures associated with representative isolates have been deposited with the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

2.2. *Morphological examination*

Asexual fruiting structures were observed on bark material sampled from the field. Growth of fruiting structures was stimulated for morphological characterization of two isolates (CMW46433 and CMW 46435) by adding freshly collected *S. cordatum* branch pieces to the culture plates. The *S. cordatum* branch sections (1.5–2 cm diameter × 5 cm length) were collected from Limpopo Province, surface sterilized, placed on the surface of 2 % water agar, and incubated at 25 ºC for 6 weeks until fruiting structures appeared. For morphological studies, fruiting structures on the original bark material collected from the field were cut from the specimens under a dissection microscope, boiled in water for 2 min and sectioned (12 µm thick) using a Leica CM1100 cryostat (set point technologies) at 120 ºC (Gryzenhout *et al*., 2004). To observe asexual structures, conidiomata were crushed on microscope slides in 85 % lactic acid or 3 % KOH. For the holotype specimen, 50 measurements were made for each of these characteristics, while 25 measurements per character were taken for the remaining specimens.

An HRc Axiocam digital camera with AXIONVISION v.3.1 software (Carl Zeiss) was used to capture images and to obtain measurements. Characteristics of specimens were compared with those published for closely related species in the Cryphonectriaceae (Gryzenhout *et al*.,

2009; Begoude *et al*., 2010; Chen *et al*., 2016; Chen *et al*., 2013; Vermeulen *et al*., 2011b). Results were presented as range of (maximum length–minimum length) × (maximum width–minimum width) (average length × average width).

Culture characteristics were studied from 2–3 representative isolates collected from different areas and hosts. These included La Réunion (CMW44151, CMW44147, CMW44126, CMW44127 and CMW44128) and South Africa (CMW46433 and CMW46435). To study culture growth, a 5 mm plug was removed from the eight cultures after 7 days of growth on 2 % MEA and transferred to the centres of 90 mm culture plates. The cultures were grown in the dark and incubated at temperatures ranging from 5 to 35 ºC at 5 ºC intervals. Five replicate plates for each isolate at each temperature were prepared. Two diameter measurements perpendicular to each other, were taken daily until the fastest growing culture had covered the plate. Averages were computed for each temperature with Microsoft Excel 2003. The entire experiment was repeated once and colour designations were obtained for the descriptions of cultures and fruiting bodies using the colour charts of Rayner (1970).

2.3. *DNA extraction, PCR amplification and DNA sequencing*

DNA was extracted from the mycelium of the 18 isolates listed in Table 1 following the methods used by Myburg *et al*. (1999). Concentrations and purity of the extracted DNA were determined with a NanoDrop 3.1.0 ND-1000uv/Vis spectrophotometer (NanoDrop Technologies, Wilmington, Delaware).

Polymerase chain reactions (PCRs) were performed following the method described by Glass and Donaldson (1995). The nuclear large subunit (LSU) and the internal transcribed spacer (ITS) regions of ribosomal DNA were amplified using primer pairs LR0R/LR5 (Vilgalys & Hester 1990) and ITS1/ITS4 (White *et al*., 1990). The β-tubulin genes were amplified using primer pairs BT1a/1b and BT2a/2bas described by Myburg *et al*. (1999; 2002a) and Chen *et al*. (2016). Sequencing reactions were performed in a volume of 12 µL consisting of 5× dilution buffer, 4.5 µL H_2O, DNA, 10X reaction mix and pmol/2 µL primer, and were cleaned using EXOSAP. The products were sequenced in both directions with the Big Dye Cycle Sequencing Kit (Applied Biosystems, Foster City, California) on an ABI PRISM^{TM} 3100 automated DNA sequencer (Applied Biosystems). 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).

2.4. *Phylogenetic analyses*

A phylogenetic species concept was used to identify the examined specimens of Cryphonectriaceae. All taxa used in phylogenetic analyses are listed in Table 1. The generic relationships in Cryphonectriaceae were analysed with a concatenated dataset of LSU, ITS and β-tubulin (including partial exon 4 and 5, partial exons 6 and 7) with taxa selected from ex-type specimens of all described genera in the Cryphonectriaceae (Begoude *et al*., 2010; Chen *et al*., 2013a; Chen *et al*., 2013b; Crane and Burgess 2013; Chen *et al*., 2016). The relationships between species were recovered by analyses of concatenated ITS and β-tubulin genes. Each gene region was analysed separately to determine whether they were phylogenetically concordant (Taylor, 2000). *Diaporthe ambigua* and *Rostrareum tropicale* were used as out-group taxa for phylogenetic analysis of genera within the Cryphonectriaceae while *Aurapex penicillata* was used as an out-group for species of *Celoporthe*.

Sequences were aligned using the iterative refinement method (FFT-NS-I settings) of MAFFT 5.667 (Katoh *et al*., 2002). The alignments were concatenated and deposited in TreeBASE (www. treebase.org.). Maximum likelihood searches for the best scoring tree were conducted with RAxML v8.2.X using the fixed General Time Reversal (GTR) model with non-parametric bootstrapping of 1000 replicates (command –f a) (Stamatakis, 2014). Bayesian analyses were performed using Mr Bayes v3.2. An MCMC analysis was run for 10 million generations with four runs consisting of four chains heated at the default temperature. Trees were sampled every 1000 generations and a 25% burn-in was used to summarize a consensus from 30,000 trees. The phylogenetic trees were viewed using Figtree.

2.5. *Pathogenicity tests*

Pathogenicity tests were conducted in a greenhouse using seven isolates from La Réunion (CMW44121, CMW44123; CMW44126; CMW44127; CMW44128, CMW44139 and CMW44147) and two isolates from South Africa (CMW46433 and CMW46435). The isolates were inoculated onto 10 trees each of *Eucalyptus grandis* clone (TAG 5) to fulfil Koch's postulates. *Eucalyptus grandis* clone TAG 5 was used as it is moderately susceptible

to species of Cryphonectriaceae. The trees were acclimatized in a greenhouse environment at 25 ºC for about a month with 14 hours of daylight prior to inoculations. The 10 isolates used for the inoculations were grown at 25 ºC under continuous fluorescent light for six days. The inoculated trees were two-years-old and 2 m tall with a main stems having diameters between 10–15 mm.

Bark discs were removed from trees with a 3 mm cork-borer to expose the cambium for inoculations. Agar disks of 3 mm diam. were cut from the margins of actively growing fungal cultures and placed into the wounds with the mycelium facing the cambium. The wounds were covered with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum plugs. A positive control, *Chrysoporthe austroafricana* (CMW2113), and a negative control of sterile MEA plugs, were each inoculated onto 10 trees. The experiment included a total of 90 inoculated trees, which were arranged randomly in the greenhouse. After 6 weeks, lesion lengths in the cambium were measured.

Lengths of the lesions on cambium were measured after bark was removed from the inoculation site. To confirm whether the isolates were responsible for the disease, pieces of necrotic tissues were randomly taken from four trees for each isolate and the inoculated trees used as controls. The pieces were placed onto the surface of 2% MEA and incubated at 25 ºC for re-isolation of the inoculated fungi. The pathogenicity trial was repeated once under the same conditions. To assess the variation in lesion length of the inoculations, means were analysed in SAS 8 with PROC GLM (general linear model) (SAS Institute, 1999). Analysis of variance (ANOVA) was conducted to determine the effects of the fungal strains on lesion length. Before ANOVA, homogeneity of variance across treatments was confirmed. Fisher's protected test was used to determine the significance among means and $P< 0.05$ for the F value taken as a significant difference.

3.0. RESULTS

3.1. *Sample collection and isolation*

In total, 26 samples resembling species in the Cryphonectriaceae with characteristic orange/yellow fruiting structures were collected from the bark of stems and branches of diseased plants. Of these, 24 samples were collected from *T. grandiflora* in La Réunion,

while one sample each was collected from native *H. natalensis* and *S. cordatum* in Limpopo, South Africa. Isolates were obtained from both sexual (ascostromata) and asexual (conidiomata) fruiting structures on MEA with various cultural characteristics ranging from fluffy white when young, which turned orange/yellow with age (Gryzenhout *et al*., 2009).

3.2. *DNA sequencing and phylogenetic analyses*

Selection of isolates for DNA sequencing and phylogenetic analyses were based on locations and hosts. For species of *Celoporthe*, twelve representative isolates collected from native *T. grandifolia* were used for the analyses, while one isolate each collected from *H. natalensis* and *S. cordatum* in South Africa were used in phylogenetic analyses for generic placement.

The number of taxa, characters and sequence length each for the new genus and species of *Celoporthe* are presented in Table 4. Phylogenetic analyses using Bayesian inference and maximum likelihood of the combined datasets of LSU, ITS and BT genes distinguished the new genus from other genera in the Cryphonectriaceae. The isolates of the new genus were distinct from other genera reported from Africa such as *Aurifilum, Diversimorbus, Holocryphia* and *Latruncellus*, but closely related to *Celoporthe* as supported by high statistical values for both maximum likehood and Bayesian analyses (ML=100, BI=1.0). This provided evidence that they belonged in a previously undescribed genus. The fungus was thus described as *Myrtonectria myrtacerum*. Although isolates of *M. myrtacerum* showed some relationship with *Immersiporthe*, *Microthia* and *Ursicollum*, however, there was no statistical evidence from the analyses to support this phylogenetic relationship using combined data sets (Fig. 2).

For *Celoporthe* species, phylogenetic analysis of the combined datasets of ITS and BT genes showed that they belong to distinct phylogenetic clades separate from other species in the *Celoporthe* such as *C. eucalypti and C. hawaiiensis*, *C. hauoliensis* and *C. syzygii,* but closely related to *C. guangdongensis* and *C. indonesiensis*. This was supported by high bootstrap values for ML and Bayesian inference (ML=76, BI=1.00) and (ML=97, BI=1.00) (Fig. 3). This evidence suggests that they represent undescribed species of *Celoporthe* and were thus named as *Celoporthe tibouchinae* and *Celoporthe borbonica*. In this study, the

newly described genus and species in the Cryphonectriaceae are distinguished by several single nucleotide polymorphisms (SNPs) (Tables 5–7).

3.3. *Morphological descriptions*

Fruiting structures observed on the cankers collected in the field for the new genus and species described in this study were predominantly asexual. The distinct orange stromatic issue showed purple staining reaction in 3% KOH, and yellow stain in lactic acid typical of species of Cryphonectriaceae (Redlin and Rossman, 1991; Gryzenhout *et al*., 2009). Teleomorph structures occurred only in *C. borbonica*, but anamorph structures occurred more abundant on the stem bark examined.

In this study, morphological characteristics distinguished the new genus from other genera and species of Cryphonectriaceae. For example, *Myrtonectria* can be distinguished from other genera in Cryphonectriaceae such as *Aurifilum*, *Celoporthe*, *Immersiporthe and Latruncellus* by its shiny dark greyish and globose to pyriform conidiomata, orange stromatic tissue and the presence of periphyses. Conidia oblong with pointed ends and orange colour in mass (Fig. 9).

Conidiomata of C. *tibouchinae* and *C. borbonica* occurring on specimens from *T. grandifolia* were completely immersed to semi-immersed, conical or hemispherical in shape, dark to brightly coloured stromata (Figs. 10 & 11), and oblong shaped conidia having sizes similar to, but differing from those of *Celoporthe* species and other species described previously in the Cryphonectriaceae. However, the most outstanding features that distinguished *C. tibouchinae* and *C. borbonica* from each other, as well as other species of *Celoporthe* are shape and size of conidiomata, and conidia size. *Celoporthe tibouchinae* had conical to hemispherical conidiomata with short necks (Fig. 10A), conidia large (4-5µm long, 2-4µm wide), oblong to ellipsoidal with pointed base (Fig. 10F). In contrast, *C. borbonica* had pulvinate to hemispherical conidiomata with cylindrical necks (Fig. 11A), conidia were oblong to allontoid in shape and small in size (3-5µm long, 1.5-3µm wide) (Fig. 11O).

Growth rate of cultures of *C. tibouchinae* and *C. borbonica* on 2 % MEA differ from those of closely related species such as *C. guangdongensis, C. indonesiensis* and *C. syzygii* as both covered 90mm plates respectively, in 6 and 8 days. The optimal growth temperature for *C. tibouchinae* and *C. borbonica* was 25 ^{o}C differing from those of *C. guangdongensis, C. indonesiensis* and *C. syzygii* which is 30^{o} C.

3.4. *Taxonomy*

Phylogenetic analyses of the combined datasets of the fungal isolates collected from *H. natalensis* and *S. cordatum* in South Africa showed distinct grouping from other genera and species in the Cryphonectriaceae thus providing evidence that belong to a new genus. Similarly, those occurring on *T. grandifolia* in La' Reunion formed two distinct and separate clades that suggest they are new species of Cryphonectriaceae. This evidence was supported by distinct morphological characteristics observed in these taxa. The new taxa are e described as follows:

3.5. ***Myrtonectria*** gen. nov. Marinc., D. B. Ali, & J. Roux

Etymology: The name refers to the fact that a fungus can kill in trees belonging to myrtaceous trees.

Sexual state not observed. *Conidiomata* semi-immersed or superficial, single or gregarious, dark greyish brown, glossy, irregular shaped or globose to pyriform, with or without protruding necks, excreting orange pigment when mounted in lactic acid and purple in KOH; *necks* cylindrical, tapering towards the apex, ostioles bright colour, *Stromatic tissue* in the middle *textura intricata*, in the inner and outer layers *textura globulosa* to *textura angularis*. *Periphyses* present near the ostiole. *Conidiophores* branched at the base, less along the length, septate, occasionally reduced to conidiogenous cells. *Conidiogenous cells* blastic, discrete, lateral or terminal, lageniform and abruptly tapering to the apex, with very narrow aperture. *Conidia* hyaline, aseptate, oblong with pointed base.

3.6. ***Myrtonectria myrtacearum*** gen. et. sp. nov. Marinc., D. B. Ali & J. Roux

Etymology: The name refers to the occurrence of this fungus on species of *Myrtaceae*.

Conidiomata on the bark, semi-immersed or superficial, single or gregarious, dark greyish brown, glossy, irregular shape or globose to pyriform, uniloculate, convoluted, with or without protruding necks, with spore droplets at the apex, 345–1340 µm long, 240–660 µm wide; *necks* cylindrical, tapering towards the apex, 240–535 µm long, 115–260 µm wide, excreting orange pigment when mounted in lactic acid and purple pigment when mounted in 2% KOH. *Stromatic tissue* at the base pseudostromatic, of *textura intricata*, at other regions eustromatic, *textura intricata* or *textura globulosa*; stromatic tissue of conidiomatal wall in the middle *textura intricata*, in the inner and outer layers *textura globulosa* to *textura angularis*, the innermost walls composed of a few layers of compressed, thin-walled cells,

the outermost walls composed of a few layers of thick-walled cells. *Periphyses* present near the ostiole. *Conidiophores* borne in a single layer along the locules, branched at the base, less along the length, septate, occasionally reduced to conidiogenous cells. *Conidiogenous cells* blastic, discrete, lateral or terminal, lageniform and abruptly tapering to the apex, with very narrow aperture, 5.5–12.5 µm long, 1.5–3 µm wide near the base. *Conidia* hyaline, aseptate, oblong with pointed base, 3–5.5 × 1.5–2 µm (avg. 3.9× 1.7 µm).

Culture characteristics: On 2% MEA colonies showing optimum growth at 25°C covering the entire 90 mm plate in 7 d, but no growth at 10°C and 35°C, mycelium flat and smooth, white when young, becoming pale to moderate yellow with orange tint at the centre.

Substrate: Bark of *H. natalensis* and *S. cordatum*.

Distribution: Limpopo province, South Africa.

Specimens examined: SOUTH AFRICA, Limpopo Province, New Agatha plantation, on bark of *Syzygium cordatum*, 29 June 2015, B. D. Ali & J. Roux (holotype PREM xxxxx, culture ex-holotype PPRI xxxxx = CMW 46433); on bark of *Heteropyxis natalensis*, 29 June 2015, B. D. Ali & J. Roux (PREM xxxxx, culture PPRI xxxxx = CMW 46435).

Notes: *Myrtonectria* can be distinguished from other genera in *Cryphonectriaceae* by its shiny dark greyish and globose to pyriform conidiomata, orange stromatic tissue and the presence of periphyses.

3.7. ***Celoporthe tibouchinae*** sp. nov: Marinc., D. B. Ali & M. J. Wingf.

Etymology: Name refers to the genus *Tibouchina*, the shrub from which the fungus was isolated.

Conidiomata immersed, erumpent, dark-coloured, single, scattered or gregarious, hemispherical or conical, 125–395 µm long, 90–400 µm wide, with an elevated ostiole (or short neck), uni- or multi-loculate, convoluted. *Stromatic tissue* in the middle often scanty but filled with reflective granules, in the innermost and outermost walls composed of compressed cells of *textura angularis*, near the apex *textura globular*. *Paraphyses* present, hyaline, simple, cylindrical, septate, 16–43 µm long, 1–2 µm wide. *Conidiophores* borne along the locular walls, branched at the base. *Conidiogenous cells* blastic, discrete, hyaline,

lageniform, 5.5–10 µm long, 1–3 µm wide. *Conidia* hyaline, aseptate, oblong to ellipsoidal, with pointed base, 2.5–4.5 × 1–1.5 µm (avg. 3.1 × 1.2 µm).

Culture characteristics: On 2% MEA colonies showing optimum growth at 30°C covering the entire 90 mm plate in 8 d, orange yellow with white margins and darker centre in reverse, showing concentric growth, mycelium flat.

Substrate: Bark of *Tibouchina grandifolia*.

Distribution: French territory and St. Joseph, La Réunion

Specimens examined: La Réunion, French territory and St. Joseph regions on bark of *Tibouchina grandifolia*, March 2015, M. J. Wingfield (holotype PREM xxxxx, ex-holotype PPRI xxxxx = CMW 44126), other cultures CMW 44127, CMW 44147.

Notes: The two undescribed species of *Celoporthe* from La Réunion were closely related and formed sister taxa to *C. indonesiensis*, *C. guangdongensis* and *C. hawaiiensis*. These species are morphologically very much alike, but the presence of reflective granules in the middle of stromatic tissue is unique to *C. tibouchineae*, which has not been reported in other species. The optimal growth temperature of *C. tibouchineae* is also similar to that of *C. guangdongensis*, *C. indonesiensis* at 30° C, whereas *C. borbonica* grew best at 25° C.

3.8. ***Celoporthe borbonica*** sp. nov: Marinc., D. B. Ali & M. J. Wingf.

Etymology: Name refers to Bourbon, the former name of La Réunion, from where the fungus was collected.

Ascostromata semi-immersed, erumpent, light colored, with necks, single or gregarious. *Stromatic tissue* prosenchymatous at the sides and the base, at the base of neck *textura angularis*. *Perithecia* sub-globose to ellipsoidal, valsoid, necks convergent, erumpent separately, periphyses along the length, peridial walls pseudoparenchymatous, dark olivaceous brown, the outer wall composed of a few layers of compressed, brown, thick-walled cells, the inner wall composed of hyaline, thin-walled cells; *necks* cylindrical, 230–395 µm long, 45–70 µm wide, *Asci* clavate to cylindrical, with non-amyloid refractive ring in apex, with deliquescent base, and lying free in the ascoma cavity, 30–42 µm long, 4.5–7 µm wide. *Ascospores* hyaline, ellipsoidal, 2-celled, septum mostly median, straight or

slightly curved; 6–10 × 2–3.5 µm (avg. 8.1 × 2.5 µm). *Conidiomata* immersed, erumpent, single, bright colored, scattered or gregarious, hemispherical or conical, uni- or multiloculate, convoluted, 280–415 µm long, 385–550 µm wide, with an ostiole: *ostioles* bright coloured. *Stromatic tissue* bright coloured. *Paraphyses* hyaline, cylindrical, septate, occasionally branched, 6.5–13.5 µm long, 3–7 µm wide. *Conidiophores* borne along the locule, branched at the base or reduced to conidiogenous cells. *Conidiogenous cells* hyaline, lageniform, 5–9.5 × 1–2 µm (avg. 7 × 1.7 µm). *Conidia* hyaline, aseptate, oblong to allantoid 2.5–4.5 × 1–1.5 µm (avg. 3.3 × 1.3 µm).

Culture characteristics: On 2% MEA colony showing optimum growth at 25° C in the dark for 7 d, limited growth at 10° C and 35° C. Mycelium buff to honey, being cinnamon at the centre, flat and smooth with even margin. Colonies white when young, turns dark with age. Colony colour the same on the reverse.

Substrate: Bark of *Tibouchina grandifolia*

Distribution: French Territory, La Réunion

Specimens examined: French territory and St. Joseph region, La' Reunion, on bark of *Tibouchina grandifolia*, March 2015, M. J. Wingfield (holotype PREM xxxxx, ex-holotype PPRI xxxxxx = CMW xxxxx); CMW 44128; other samples CMW 44139, CMW 44144.

Note: The asexual morph of *C. borbonica* is very similar to that of *C. tibouchinae*. *Celoporthe borbonica* has a lower optimal growth temperature (25°C) than that of *C. tibouchinae* (25°C). In comparison with other species of *Celoporthe* that produce a sexual morph, *C. borbonica* has slightly larger ascospores (6–10 × 2–3.5 µm) than that of *C. dispersa* (4.5–8 × 2–3.5 µm) and *C. syzygii* (5–8.5 × 2.5–3.5 µm).

3.9. *Pathogenicity test*

After 6 weeks of inoculation, all the isolates used in the trials caused lesions on the stem of *Eucalyptus* clone (TAG 5), but none was observed for the control (Fig. 13). The mean comparison tests showed that the average lesion length caused by two isolates of

Myrtonectria myrtacerum, three isolates of *C. tibouchineae* and *C. lareunionensis*, and one isolate of *Chrysoporthe austroafricana* were all significantly longer ($p<0.001$) than the wounds caused by the negative control (Table 10). This was similar in the second experiment. Isolates CMW46433, CMW44123 and CMW44126 which represented *M. myrtacerum*, *C. lareunionensis* and *C. tibouchineae* respectively, were more aggressive than the rest of the isolates (Fig. 12). However, these isolates are less aggressive compared to *Chr. austroafricana* that kill a few seedlings during the period of the experiment.

4.0. DISCUSSION

This study used a combined morphological and molecular approach to further document cryptic biodiversity of fungi in the Cryphonectriaceae that exist as endophytes and canker forming pathogens on species of Myrtaceae. A newly described taxon, *Myrtonectria myrtacearum*, was discovered on diseased *H. natalensis* and *S. cordatum* trees in New Agatha, Limpopo Province, South Africa. *Myrtonectria* was described for its shiny dark greyish and globose to pyriform conidiomata and is mildly pathogenic on species of *Eucalyptus*. It is one of 15 monotypic genera so far described in the Cryphonectriaceae and the fifth of these genera described from South Africa.

Two new species of *Celoporthe* were described from La Réunion on diseased branches of native *T. grandifolia* trees as *C. tibouchinae* and *C. borbonica*. There are now 11 species of *Celoporthe* described from Melastomataceae and Myrtaceae. These are the first species of *Celoporthe* reported from La Réunion, and the ninth species of *Celoporthe* so-far described from Myrtales in Africa and Asia (Nakabonge *et al*., 2006a, Chen *et al*., 2011, Vermeulen *et al*. 2013).

Celoporthe was hypothesised to have diversified in Africa and Asia because isolates from these countries formed two monophyletic groups that corresponded to their country of origin (Nakabonge *et al*. 2006a). However, Chen *et al*. (2011) observed that *Celoporthe* might have been introduced into different regions of Asia where they have been found. The observation

was based on the fact that all hosts infected by *Celoporthe* species are non-native to these areas (Chen *et al.* 2011).

Myrtonectria myrtacearum, *C. dispersa*, *C. fontana* and *C. woodiana* have a similar host range in South Africa, all endophytic in *Heteropyxis* and *Syzygium cordatum* (Nakabonge *et al*. 2006b). This shows that a large number of fungi occupy one environmental niche as endophytes, and highlights that movement of plant material, especially Myrtaceae, poses a risk of introducing possible pathogens to new areas.

The pathogenicity trials of the new taxa showed these were all mildly pathogenic on *Eucalyptus*. They may be capable of shifts from their native range to *Eucalyptus* trees used in forestry plantations. Movement of these pathogens to new environments through agriculture or forestry is a biosecurity concern. A similar situation occurred with the case of *Cryphonectria parasitica* that was moved together with chestnut seedlings from Asia to North America where it ravaged *Castanea dentata* trees in their natural ranges (Anagnostakis, 1987).

The discovery and description of *M. myrtacearum*, *C. tibouchinae* and *C. borbonica* from native Myrtales in South Africa and La Réunion in this study, and the recent discovery of *Corticirmorbus sinomytri* and *Lagerstroemia speciosa* also from native Myrtales in China (Chen *et al*. 2016, 2017) shows there is undiscovered biodiversity of cryptic species of Cryphonectriaceae. All of these recently described species infected *Eucalyptus* during greenhouse inoculation trials, and highlight the importance and risk endophytic fungi must have in biosecurity awareness.

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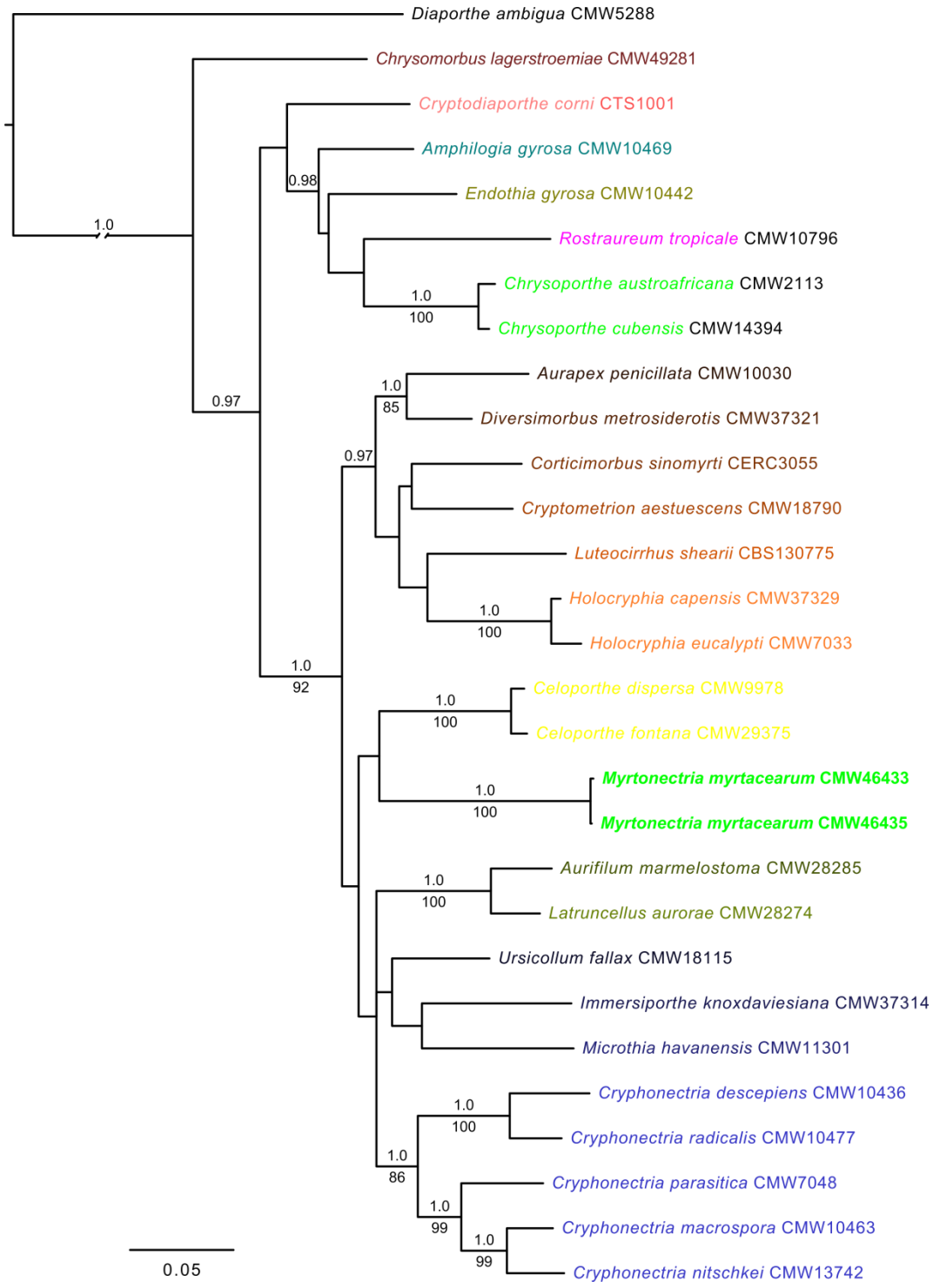

Fig.2: A phylogenetic tree of Maximum likelihood (ML) and Bayesian analyses of a combined data sets of the 28S regions of LSU, 5.8S rDNA and partial exon 4, and exon 5, and partial exon 6 and 7 of the BT 1 and BT2 genes. Statistical bootstrap values >70 and 0.7 for ML analysis are shown at the nodes, while Bayesian inference is indicted with a bold branches, while absent analyses are represented by–. Isolates of the new genus are in bold face and highlighted.

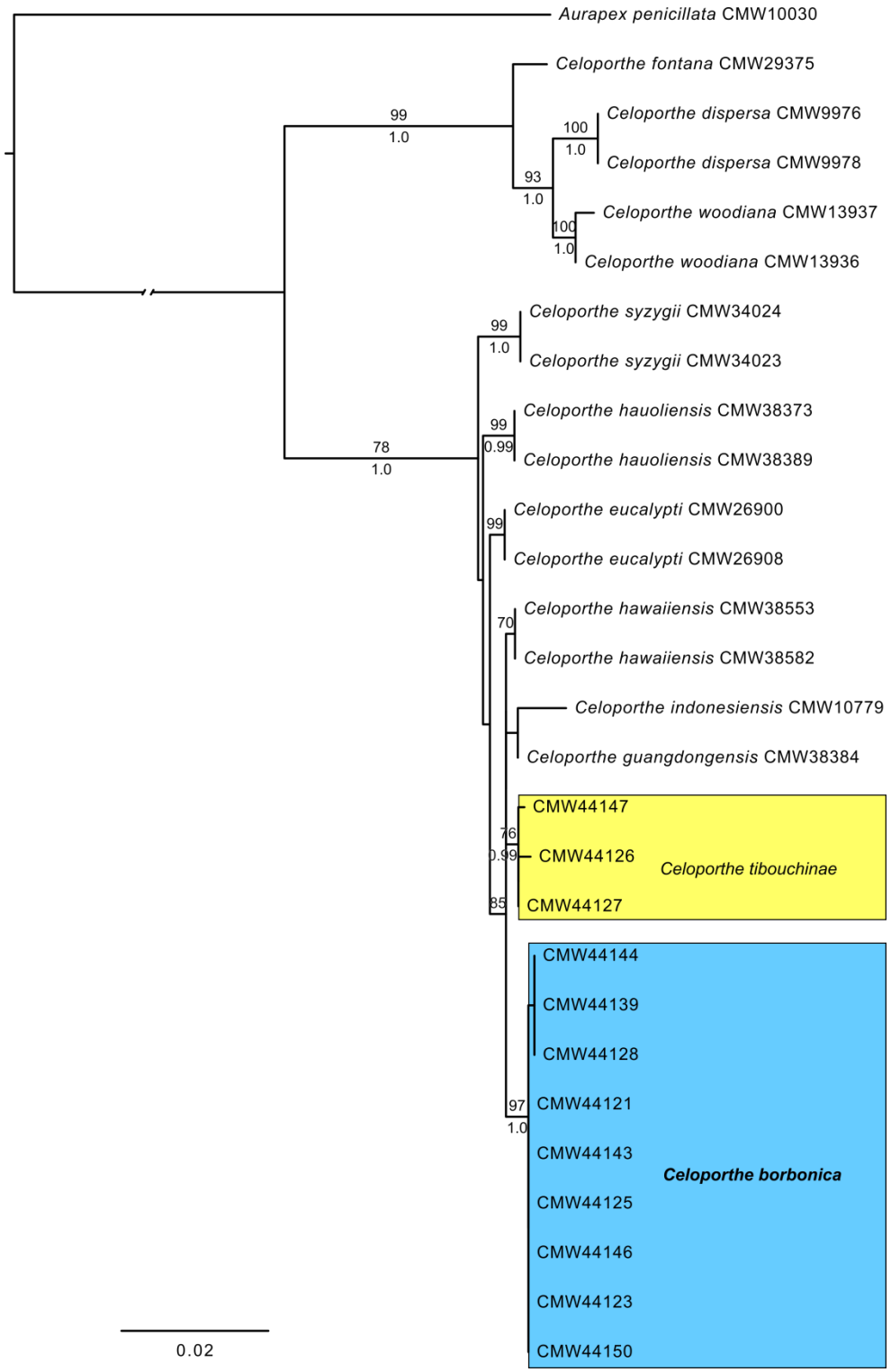

Fig. 3: A phylogenetic tree of Maximum likelihood (ML) and Bayesian analyses of a combined data sets of the 5.8S rDNA and partial exon 4 and exon 5, and partial exon 6 and 7 of the BT1 and BT2 genes. Statistical bootstrap values >70 and 0.7 respectively for ML analysis and Bayesian inference are shown on the nodes. Isolates of the new species are in bold face and highlighted.

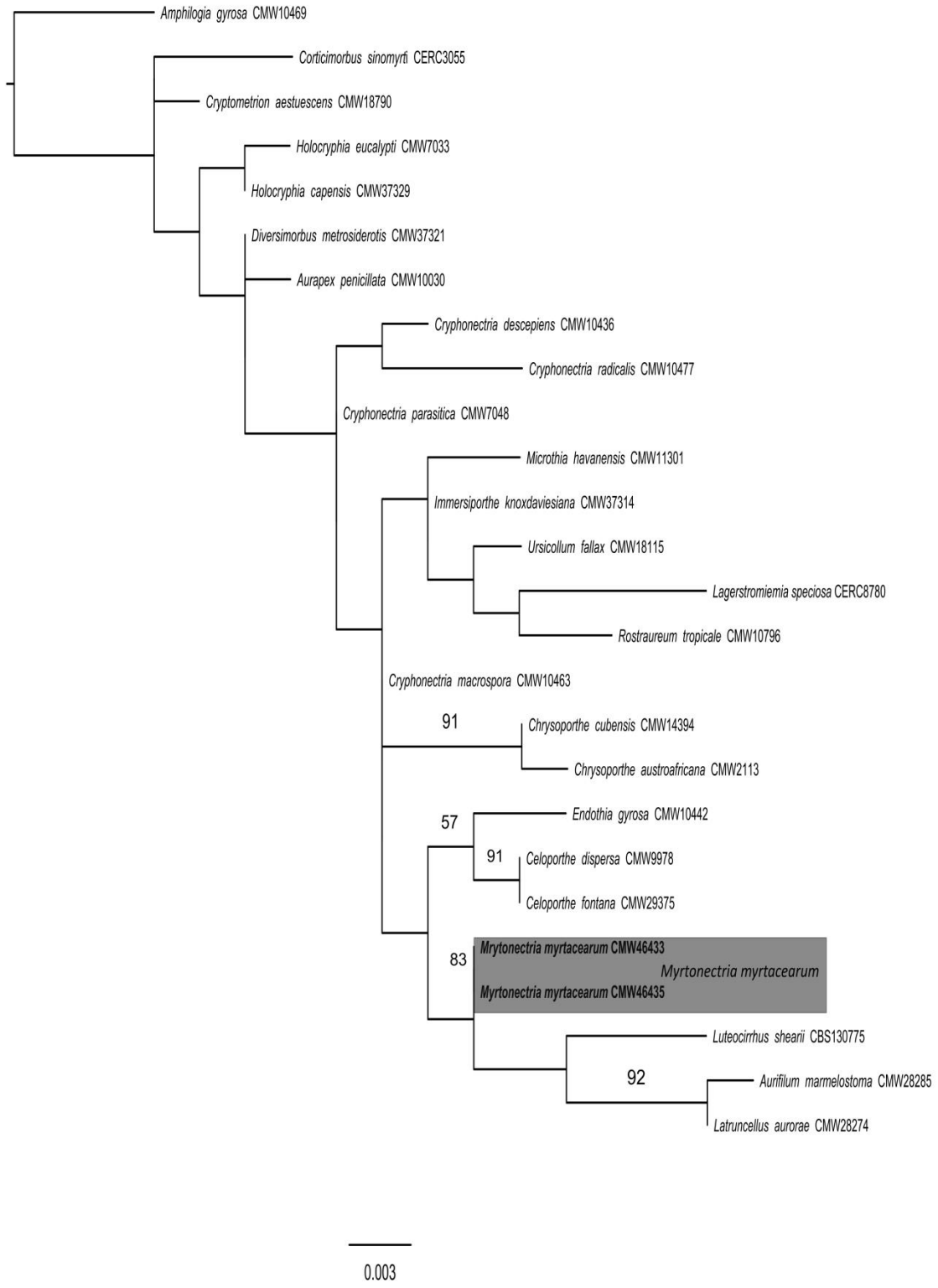

Fig. 4: A phylogenetic tree of Maximum likelihood (ML) of the 5.8S rDNA large subunit. Statistical bootstrap values >70 for ML analysis are shown on the nodes, while absent analyses are represented by–. Isolates of the new species are in bold face and highlighted.

Fig. 5: A phylogenetic tree of Maximum likelihood (ML) of the ITS including the 5.8S rDNA gene. Statistical bootstrap values >70 for ML analysis is shown on the nodes, while absent analyses are represented by–. Isolates of the new genus and species are in bold face and highlighted.

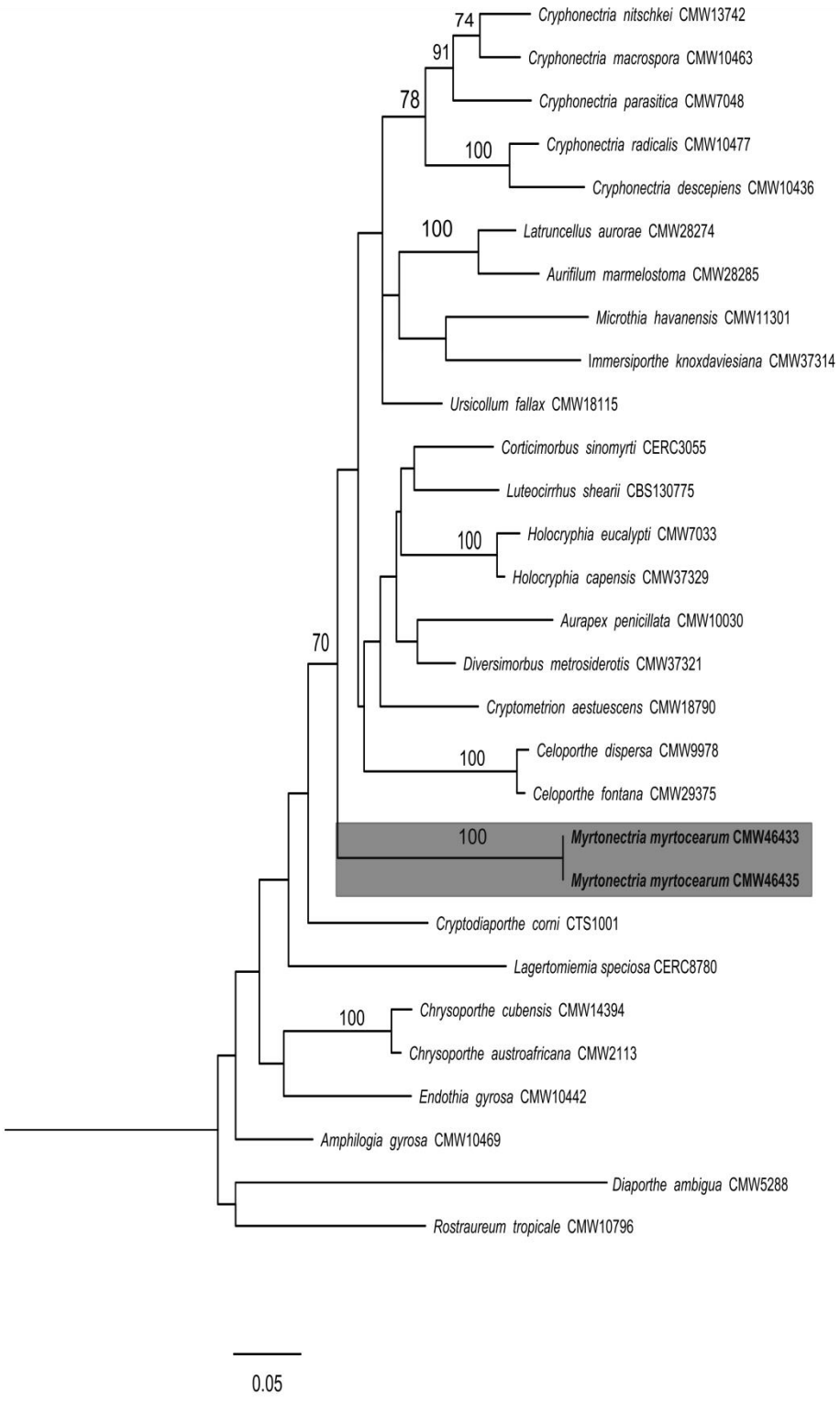

Fig. 6: A phylogenetic tree of Maximum likelihood (ML) analyses of partial exon 6 and 7 of the BT1 gene for the new genus. Statistical bootstrap values >70 for ML analysis are shown on the nodes, while absent analyses are represented by–. Isolates of the new species are in bold face and highlighted.

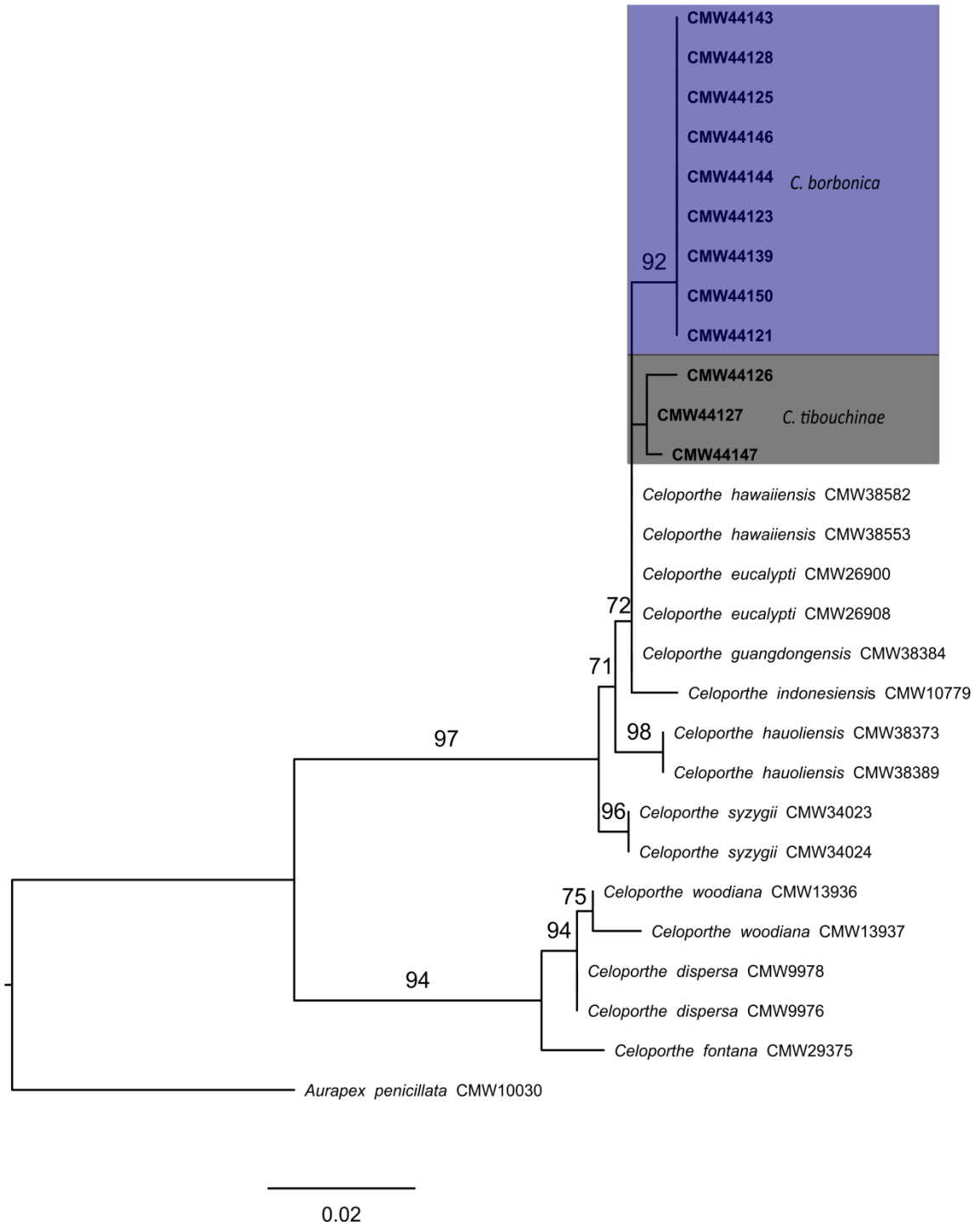

Fig. 7: Phylogenetic tree of Maximum likelihood (ML) analyses of ITS including the 5.8S rDNA gene. Statistical bootstrap values >70 for ML analysis are shown on the nodes. Isolates of the new species are in bold face and highlighted.

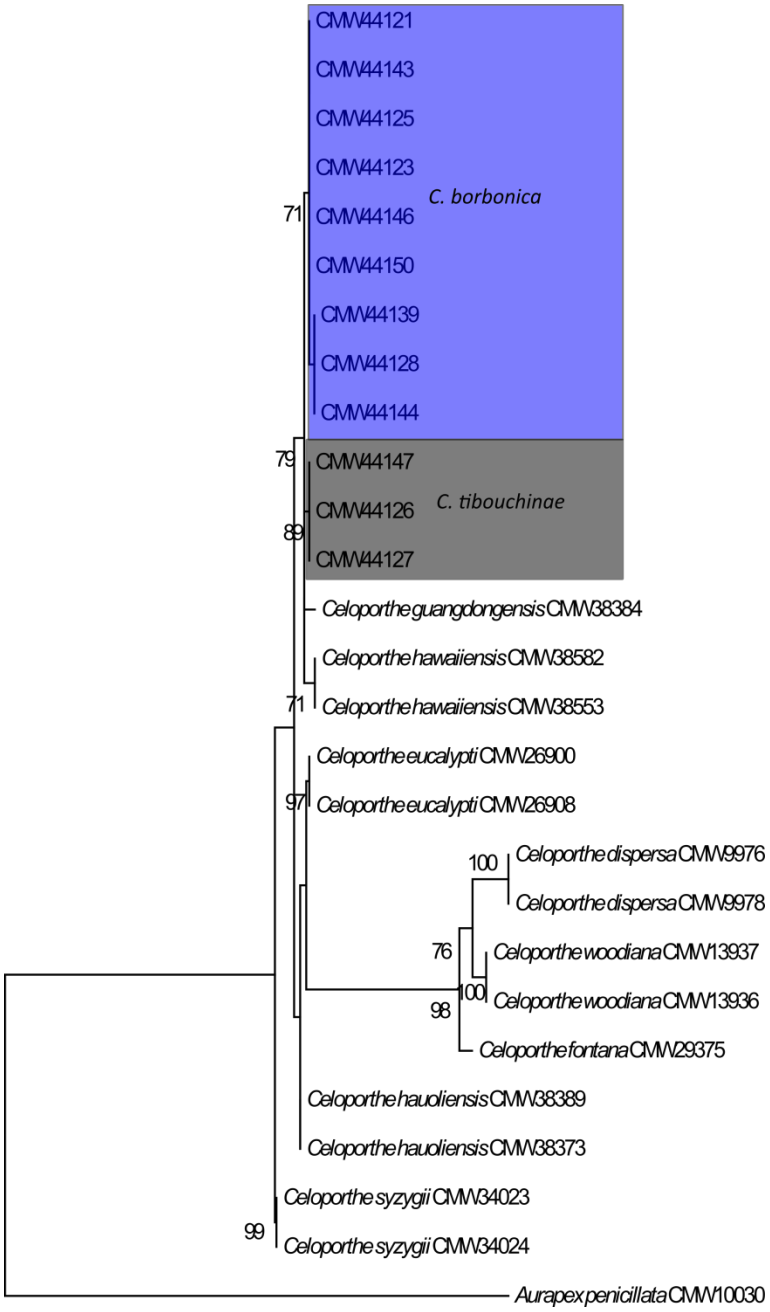

Fig. 8: Phylogenetic tree of Maximum likelihood (ML) analyses of partial exon 6 and 7 of BT1. Statistical bootstrap values >70 for ML analysis are shown on the nodes. Isolates of the new species are in bold face and highlighted.

TABLE 4: ISOLATES OF CRYPHONECTRIACEAE USED IN PHYLOGENETIC STUDIES AND PATHOGENICITY STUDY

Identity	***Isolate no.***[a]	***Host***	***Location***	***Collector***	***GenBank accession no.***[b]				***References***
					LSU	***ITS***	***BT1***	***BT2***	
Amphologia gyrosa	CMW10469	*Elaecarpus dentata*	New Zealand	G. J. Samuels	AY194107	AF452111	AF525707	AF525714	Gryzenhout *et al*. (2005a, 2006e)
	CMW10470	*E. dentatus*	New Zealand	G. J. Samuels	AY194108	AY452112	AF525708	AF525715	Gryzenhout *et al*. (2005a, 2006e)
	CTS1001	N/A	USA	K. Kitka	N/A				Beier *et al*. (2014)
Aurapex penicillata	CMW10030	*Microthia theaezens*	Colombia	C.A Rodas	AY194103	AY214311	AY214239	AY214275	Gryzenhout *et al*. (2006c, 2009)
Aurifilum marmelostroma	CMW28285	*Terminalia mantaly*	Cameroon	D. Begoude & J. Roux	HQ171215	FJ882855	FJ900585	FJ900590	Begoude *et al*. (2010)
Celoporthe dispersa	CMW9976	*Syzygium cordatum*	*South Africa*	M. Gryzenhout	HQ730853	DQ267130	DQ267136	DQ267142	Nakabonge *et al*. (2006)
	CMW9978	*S. cordatum*	*South Africa*	M. Gryzenhout	HQ730852	DQ267136	DQ267142	AY214316	Nakabonge *et al*. (2006)
C. eucalypti	CMW26900	*Eucalyptus clone EC48*	China	X. D Zhou & S.F. Chen	HQ730862	DQ267136	HQ730816	HQ730826	Chen *et al*. (2011)
	CMW26908	*EucalyptusEC 48 clone*	China	X. D Zhouu & S.F. Chen	HQ730863	HQ730837	HQ730817	HQ730827	Chen *et al*. (2011)
C. fontana	CMW29375	*Syzygium guineense*	Zambia	M. Vermeulen & J. Roux	N/A	GU726940	GU726952	GU726952	Vermeulen *et al.* (2013b)

TABLE 4: CONTINUED

Identity	*Isolate no.*[a]	*Host*	*Location*	*Collector*	*GenBank accession no.*[b]				**References**
					LSU	***ITS***	***BT1***	***BT2***	
C. fontana	CMW29376	*S. guineense*	Zambia	M. Vermeulen & J. Roux	N/A	GU726941	GU726953	GU726953	Chen *et al*. (2011)
C. guangdongensis	CMW12750	*Eucalyptus sp.*	China	T. I. Burgess	*HQ730856*	HQ730830	HQ730810	HQ730820	Chen *et al*. (2011)
C. *indonesiensis*	CMW10781	*S. aromaticum*	Indonesia	M. J. Wingfield	HQ730855	AY084009	AY084033	AY084021	Chen *et al*. (2011)
C. syzygii	CMW34023	*Syzygium cumini*	China	S. F. Chen	HQ730857	HQ730831	HQ730811	HQ730821	Chen *et al*. (2011)
	CMW24912	*S. cumini*	China	S. F. Chen	HQ730859	HQ730833	HQ730813	HQ730823	Chen *et al*. (2011)
C. woodiana	CMW13936	*Tibouchina granulosa*	*South Africa*	*M. Gryzenhout*		DQ267131	DQ267137	DQ267143	Vermeulen *et al.* (2013b)
C. woodiana	CMW13937	*T. granulosa*	*South Africa*	*M. Gryzenhout*		DQ267132	DQ267138	DQ267144	Vermeulen *et al.* (2013b)
C. tibouchinae	CMW44126	*T. grandiflora*	La' Reunion	M. J. Wingfield	N/A	**MG585747**	**MG585731**	N/A	***In this study***
C. tibouchinae	CMW44127	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A	**MG585748**	**MG585732**	N/A	***In this study***
C. tibouchnae.	CMW44147	*T. grandiflora*	La Réunion	M. J. Wingfield	**N/A**	**MG585749**	**MG585733**	N/A	***In this study***
C. borbonica	CMW44121	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A	**MG585741**	**MG585725**	N/A	***In this study***
C. borbonica	CMW44123	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A				
C. borbonica	CMW44128	*T. grandifora*	La Réunion	M. J. Wingfield	N/A				

TABLE 4: CONTINUED

Identity	*Isolate no.*[a]	*Host*	*Location*	*Collector*	*GenBank accession no.*[b]				**References**
					LSU	***ITS***	***BT1***	***BT2***	
C. borbonica	CMW44144	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A	MG585744	**MG585728**	**N/A**	***In this study***
C. borbonica	CMW44146	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A	MG585745	**MG585729**	**N/A**	***In this study***
C. borbonica	CMW44150	*T. grandiflora*	La Réunion	M. J. Wingfield	N/A	MG585746	**MG585730**	**N/A**	***In this study***
Chrysomorbus lagerstroemiae	CMW49281	*Lagertroemia speciosa*	China	J. Roux & S. F. Chen	KY929320	KY929330	KY929350	KY929340	Chen *et al*. (2017)
Chrysoporthe austroafricana	CMW9327	*Tibouchina granulosa*	South Africa	J. Roux	N/A	GQ290158	GQ290185	GQ290194	Gryzenhout *et al.* (2006c; Myburg *et al*. 2002b)
Chrysoporthe austroafricana	CMW2113	*Eucalyptus gransis*	South Africa	M. J. Wingfield	JN940852	AF 046892	AF 273067	AF 273462	Gryzenhout *et al.* (2006c); Myburg *et al*. 2002b).
Chr. cubensis	CBS118654	*Eucalyptus*	Colombia			DQ368773	AH015642	AH015642	Gryzenhout *et al*. (2006b).
Chr. cubensis	CMW10028	*Eucalyptus*	Colombia	M. J. Wingfield	JN940856	GQ290153	GQ290175	GQ290186	Gryzenhout *et al*. (2004).
Chr. deuterocubensis	CMW2631	*S. aromaticum*	Indonesia	M. J. Wingfield	N/A	GQ290157	GQ290184	AF543825	Gryzenhout *et al*. (2004).
Chr. deuterocubensis	CMW8650	*Melastoma*	Indonesia	M. J. Wingfield	KY929320	KY929330	AY084024	GQ290193	Gryzenhout *et al*. (2004).

TABLE 4: CONTINUED

Identity	*Isolate no.*[a]	*Host*	*Location*	*Collector*	*GenBank accession no.*[b]				References
					LSU	*ITS*	*BT1*	*BT2*	
Chrysoporthe doradensis	CMW11286	*E. grandis*	Ecuador	M. J. Wingfield	JN940845	AY214289	AY214217	AY214253	Gryzenhout *et al*. (2005a)
	CMW11287	*E. grandis*	Ecuador	M. J. Wingfield	JN938748	GQ290156	GQ290179	GQ290190	Gryzenhout *et al*. (2005a)
Chrysoporthe hodgesiana	CMW9995	*T. semidecandra*	Colombia	R. Arbalaez		AY956969	AY956978	AY956977	Rodas *et al*. (2005)
	CMW10625	*M. theaezans*	Colombia	C. A. Rodas	JN940842	AY956970	AY956979	AY956980	Rodas *et al*. (2005)
Chrysoporthe inopina	CMW12727	*T. lepidoa*	Colombia	R. Arbalaez		DQ368777	GQ290180	DQ368806	Gryzenhout *et al*. (2006b)
Chrysoporthe doradensis	CMW11286	*E. grandis*	Ecuador	M. J. Wingfield	JN940845	AY214289	AY214217	AY214253	Gryzenhout *et al*. (2005a)
	CMW11287	*E. grandis*	Ecuador	M. J. Wingfield	JN938748	GQ290156	GQ290179	GQ290190	Gryzenhout *et al*. (2005a)
	CMW12731	*T. lepidota*	Colombia	R. Arbalaez		DQ368779	GQ290182	DQ368811	Gryzenhout *et al*. (2006b)
Chrysoporthe syzygiicola	CMW29940	*Syzygium guineense*	Zambia	D. Chungu & Roux		FJ655005	FJ805230	FJ805236	Chungu *et al*. (2010)
	CMW29941	*S. guineense*	South Africa	D. Chungu & Roux		FJ655006	FJ805231	FJ805237	Chungu *et al*. (2010)
Chr. zambiensis	CMW29928	*Eucalyptus grandis*	Zambia	D. Chungu & Roux		FJ655002	FJ858709	FJ805233	Chungu *et al*. (2010)
	CMW29929	*E. grandis*	Zambia	D. Chungu & Roux		FJ655003	FJ858710	FJ805234	Chungu *et al*. (2010)

TABLE 4: CONTINUED

Identity	*Isolate no.*[a]	*Host*	*Location*	*Collector*	*GenBank accession no.*[b]				References
					LSU	*ITS*	*BT1*	*BT2*	
Chr. syzygiicola	CMW29940	*Syzygium guineense*	Zambia	D. Chungu & Roux		FJ655005	FJ805230	FJ805236	Chungu *et al*. (2010)
Corticimobus sinomyrti	CERC 3055	*Rhodomyrtus tomentosa*	China	S. F. Chen & G. Q. Liu	KT167172	KT167162	KT167182	KT167182	Chen *et al*. (2016)
Cryphonectria decipiens	CMW10436	*Quercus suber*	Portugal	B. d'Oliviera	JQ862750	AF452117	AF525703	AF525710	Myburg *et al.* (2004b)
C. nitschkei	CMW13742	*Quercus grosseserrata*	Japan	T. Kobayashi	N/A	AY697936	AY697961	AY697962	Myburg *et al*. (2004b)
C. macrospora	CMW10463	*Castanea cuspidata*	Japan	T. Kobayashi	N/A	AF368331	AF368351	AF368350	Gryzenhout *et al*. (2006c)
C. parasitica	CMW7048	*Quercus virginiana*	USA	R. Stipes	AY194100	AF368330	AF273076	AF273470	Gryzenhout *et al.* (2006a) Venter *et al*. (2001)
C. radicalis	CMW10477	*Q. suber*	Italy	A. Braghi	AY194102	AF368328	AF368347	AF368347	
Cryptometrion aestuescens	CMW18790	*Eucalyptus grandis*	Indonesia	M. J. Wingfield	HQ171211	GQ369458	GQ369455	GQ369455	Gryzenhout *et al*. (2010)
Diversimorbus metrosiderotis	CMW37321	*Metrosideros angustifolia*	South Africa	J. Roux	JQ862827	JQ862870	JQ862911	JQ862952	Gryzenhout *et al*. (2010)
Holocryphia capensis	CMW37329	*M. angustifolia*	South Africa	J. Roux and S. F. Chen	JQ862816	JQ862859	JQ862900	JQ862941	Chen *et al*. (2013)
H. eucalypti	CMW7033	*Eucalyptus grandis*	South Africa	M. Venter	JQ862794	JQ862837	JQ862878	JQ862919	Chen *et al*. (2013b)
H. capensis	CMW37329	*M. angustifolia*	South Africa	J. Roux and S. F. Chen	JQ862816	JQ862859	JQ862900	JQ862941	Chen *et al*. (2013)

TABLE 4: CONTINUED

Identity	*Isolate no.*[a]	*Host*	*Location*	*Collector*	*GenBank accession No*				**References**
					LSU	***ITS***	***BT1***	***BT2***	
Immersiporthe knoxdaviesiana	CMW37314	*Rapanea melanophiloeos*	South Africa	M. J. Wingfield & J. Roux	JQ862755	JQ862765	JQ862785	JQ862775	Chen *et al*. (2013)
Latruncellus aurorae	CMW28274	*Galpinia transvaalica*	Swaziland	J. Roux	HQ171213	GU726946	GU726958	GU726958	Vermeulen *et al*. (2011)
Luteocirrhus shearii	CBS 130775	*Bankesia baxteri*	Australia	C. Crane	KC197018	KC197024	KC197015	KC197009	Crane & Burgess (2013)
Myrtonectria myrtacearum	CMW8650	*S. aromaticum*	Indonesia	M. J. Wingfield	N/A	AY084001	AY084024	GQ290193	van der Merwe *et al*. (2009)
	CMW46433	*S. cordatum*	South Africa	D.B. Ali & J. Roux	**MG585750**	**MG585736**	**MG585720**	**MG585734**	**In this study**
M. myrtacearum	CMW46435	*H. natalensis*	South Africa	D.B. Ali & J. Roux	**MG585751**	**MG585737**	**MG585721**	**MG585735**	**In this study**
Rosraureum tropicale	CMW10796	*Terminalia ivorensis*	Ecuador	M. J. Wingfield	N/A	AY167438	AY167428	AY167433	Gryzenhout *et al*. (2005c)
Ursicollum fallax	CMW18115	*Coccoloba uvifera*	USA	C. S. Hodges	N/A	DQ368756	DQ36860	DQ368761	Gryzenhout *et al*. (2006d)

Table 5: Summary of polymorphic nucleotides found within sequences of the ribosomal ITS region and BT genes for all Asian clade of *Celoporthe* species including the new species described in this study. Polymorphic nucleotides unique to each species are shown in bold face.

	ITS2/5.8S/ITS4																			
Taxon	**8**	**24**	**25**	**27**	**30**	**33**	**73**	**75**	**84**	**86**	**88**	**101**	**113**	**118**	**306**	**340**	**347**	**432**	**433**	**435**
A. marmelostroma CMW 28285	C	-	C	G	G	-	G	T	G	A	G	G	A	T	C	C	G	T	C	T
Celoporthe disersa CMW9978	T	C	G	T	T	G	G	T	G	A	G	A	A	A	C	A	A	C	T	-
CMW 46433	C	-	C	G	A	-	T	G	A	C	C	G	T	A	T	A	G	C	T	-
CMW 46435	C	-	C	G	A	-	T	G	A	C	C	G	T	A	T	A	G	C	T	-

	BT1						BT2			
	212	**236**	**254**	**257**	**284**	**305**	**14**	**79**	**175**	**283**
A. marmelostroma CMW 28285	G	T	G	C	C	C	T	T	A	T
Celoporthe disersa CMW9978	G	C	A	G	T	G	C	T	G	C
CMW 46433	A	C	G	C	C	C	T	C	G	C
CMW 46435	A	C	G	C	C	C	T	C	G	C

Table 6: Summary of polymorphic nucleotides found within sequences of the ribosomal ITS region and BT genes for all Asian clade of *Celoporthe* species including the new species described in this study. Polymorphic nucleotides unique to each species are shown in bold face.

TAXON	ITS/5.8S/ITS2											BT1						
	19	**60**	**62**	**188**	**189**	**195**	**406**	**416**	**464**	**465**	**466**	**54**	**102**	**128**	**117**	**188**	**191**	**199**
CMW 44147	C	-	**G**	T	A	**C**	G	A	T	G	-	C	G	T	A	C	T	A
CMW 44127	C	-	**G**	T	A	**A**	G	A	T	G	-	C	G	T	A	C	T	A
CMW44126	C	-	**G**	T	A	**A**	G	A	T	G	-	C	G	T	A	C	T	A
CMW44128	C	**G**	A	**A**	**C**	-	G	A	T	G	**T**	C	G	T	**G**	C	T	A
CMW44139	C	**G**	A	**A**	**C**	-	G	A	T	G	**T**	C	G	T	**G**	C	T	A
Cel. indonesiensis CMW10779	**T**	A	A	T	A	-	C	**G**	T	G	-	-	-	-	-	-	-	-
Cel. hawaiiensis CMW38582	C	-	A	T	A	-	G	A	**-**	-	-	C	G	**G**	A	C	T	A
Cel. guandongensis CMW12750	C	-	A	T	A	-	G	A	T	G	-	**T**	G	T	A	C	T	**C**
Cel. eucalypti CMW26900	C	-	A	T	A	-	G	A	T	G	-	C	A	T	A	**T**	**C**	A

Table 7: Statistics resulting from phylogenetic analysis

Analysis	*M. myrtacerum*					*C. tibouchineae*				*C. lareunionensis*		
	LSU	ITS	BT1	BT2	Combined	ITS	BT1	BT2	Combined	ITS	BT1	Combined
Sequence length (bp)	591	582	417	364	-	542	412	383	-	529	484	
No. of taxa	71	99	71	71	29	27	28	-	31	27	28	31
No. of characters (bp).	531	617	398	315	1684	-	-	-	1320	-	-	1320
ML Rate matrix												
Bayesian Rate matrix												
Tree length												

Table 8: Comparisons of *Celoporthe* species described so far

Parameter		*C. dispersa*	*C. eucalypti*	*C. guangdongensis*	*C. indonesiensis*	*C. syzygii*	*Celoporthe tibouchii*	*Celoporthe lareunioensis*
Collected from	country	South Africa	China	China	Indonesia	China	La' Reunion	La' Reunion
	host	*S. cordatum*	*S. cumini*	*Eucalyptus* sp.	S. *aromaticum*	*S. cumini*	*T. grandifolia*	*T. grandifolia*
Conidiomata	height	**300–500**[a]	**80–400(av. 343)**	**90–720 (av. 255)**	**60–260 (av. 110)**	**80–400 (av. 163)**		
	shape		**Pulvinate sometimes conical**	**Pulvinate sometimes conical without necks**	**Pulvinate**	**Pulvinate without necks**	**Conical to hemispherical with short necks**	**Pulvinate to hemispherical cylindrical necks**
Optimal growth temp.			**30 ^{O}C**	**30 ^{O}C**	**30 ^{O}C**	**30 ^{O}C**	**25 ^{O}C**	**25 ^{O}C**
(above the bark)	width	**200–1000**	**140–700(av.370)**	**120–750 (av.395)**	**100–550 (av.225)**	**140–900 (av. 343)**		
Conidiogenous cells	width	(1.5–)2.0–3.0	1.5–2.5(–3.5) (av. 2.2)	1.5–2.5 (–3.5) (av. 2.0)	1.0–3.5 av. (3.5) (av. 2.0)	1.0–2.0(–2.5) (av. 2.0)		
Paraphyses or sterile cells	length	**up to 39(av. 20)**	**up to 68 (av. 42)**	**up to 91(av. 55)**	**up to 91(av. 55)**	**up to 52 (av. 20)**		
Conidia	shape	Oblong, cylindrical, to occasionally allantoid	Oblong, cylindrical to occasionally allantoid	Oblong, cylindrical to occasionally allantoid	Oblong Cylindrical, or allantoid	Oblong, cylindrical to occasionally allantoid	Oblong to ellipsoid with pointed base	oblong to allantoid
	length	2.5–5.5 (av.3.5)	2.6–4.4 (av.3.5)	2.4–4.3 (av.3.5)	3.1–4.7 (av.3.9)	2.3–3.8 (av.3.1)	4–6.5 (ave. 5.21)	3–5 (ave 3.84)
	width	1.0–2.5 (av. 1.5)	1.1–1.8 (av. 1.6)	1.1–1.9 av. 1.5	1.1–1.6 (av. 1.3)	1.0–1.6 (av.1.3)	2–4 (ave. 2.75)	1.5–3 (ave. 1.84)

[a]The measurements are in micrometre, and bold face indicates distinctive characters; n/a, not available

Table 8: Continued

Parameter		*C. dispersa*	*C. eucalypti*	*C. guangdongensis*	*C. indonesiensis*	*C. syzygii*	*Celoporthe tibouchineae*	*Celoporthe lareunioensis*
Ascostromata (above the neck)	height	100–400[a]	40–240(av.120)	n/a	n/a	n/a	n/a	pulvinate or hemispherical with protruding necks
	width	320–505	280–580 (av.300)	n/a	n/a	n/a	n/a	?
Perithecia	no.of perithecia per ascostromata	**1–6**	**1–15**	n/a	n/a	n/a	n/a	?
	diameter	**100–300**	**60–380 (av. 170)**	n/a	n/a	n/a	n/a	?
Perithecial necks	length	**50**	**40**	n/a	n/a	n/a	n/a	?
	width	**100–150**	**40-90**	n/a	n/a	n/a	n/a	?
Asci	shape	fusoid to ellipsoidal	**fusoid**	n/a	n/a	n/a	n/a	clavate to ellipsoid
	length	**19.5–33.5 (av. 26.5)**	**29.5–43 (av. 34.0)**	n/a	n/a	n/a	n/a	**22–38 (ave. 30.74)**
	width	**4.5–7.5 (av.6.3)**		n/a	n/a	n/a	n/a	**5–10 (ave. 7.28)**
Ascospores	shape	oblong ellipsoidal	oblong ellipsoidal	n/a	n/a	n/a	n/a	Clavate to to ellipsoidal
	length	4.5–8 (av. 6.5)	5–8.5 (av. 6.8)	n/a	n/a	n/a	n/a	7–13 (ave. 9.40)
	width	2–3.5 (av. 2.8)	2.5–3.5 (av. 2.8)	n/a	n/a	n/a	n/a	3–7 (ave. 4.35)

[a]The measurements are in micrometre, and bold face indicates distinctive characters; n/a, not available

Table 9: Morphological characteristics of *Myrtonectria myrtacerum* compared with other genera of Cryphonectriaceae having entirely black conidiomata

Morphological characteristics		*Aurifilum*	*Celoporthe*	*Immersiporthe*	*Latruncellus*	*Myrtonectria myrtacerum*
Teleomorph	Structure of ascostromata	Large, pulvinate to pyriform, semi immersed	Valsoid	Not observed	Pulvinate	Not known
	Asci		Fusoid to ellipsoid	Not observed	Fusoid to ellipsoid	Not known
	Ascospores shape	Oblong to ellipsoid to with rounded ends	Oblong to ellipsoid	Not observed	Fusoid to oval	Not known
	Ascospores septation	One septate	One septate	Not observed	One septate	Not known
Anamorph	Colour of conidiomata when mature	Uniformly black	Fuscous black	Umber to brown	orange	Pale
	Conidiomatal position in bark	Usually beneath or erumpent, semi-immersed	Superficial to slightly immersed	Immersed to semi-immersed	Semi-immersed	Semi-immersed, erumpent
	Conidiomata shape	Pulvinate to pyriform,	Pulvinate to conical	Pulvinate	Conical	Pulvinate to triangular with
	Conidiomatal necks	Absent, ostiolar opening darkened	Absent or with short attenuated necks	Absent	Neck restricted and flattened	protruding necks
	Paraphyses	Present		Present	Present	Absent
	Conidial shape	Cylindrical or allantoid	Oblong to cylindrical to ovoid	Cylindrical to fusoid occasionally allontoid	Minute, cylindrical	Oblong with pointed base
	Conidial size	(3.0–)[a] 3.5–4.5 (–5.0) 9 1.0–1.5 (–2.5)	(2.5–)3–4(–5.5) × (1–)1.5(–2.5)		4(–4.5) × 1–1.5 (–2)	
	Conidial colour in mass	Orange	Bright luteous	Orange	Orange	Orange

[a] The measurements are in micrometre, and bold face indicate distinctive characters; n/a, not available.

Table 10: Analysis of Variance (ANOVA)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	11	51945,9	4722,4	27,73	<.001
Residual	108	18391,7	170,3		
Total	119	70337,6			

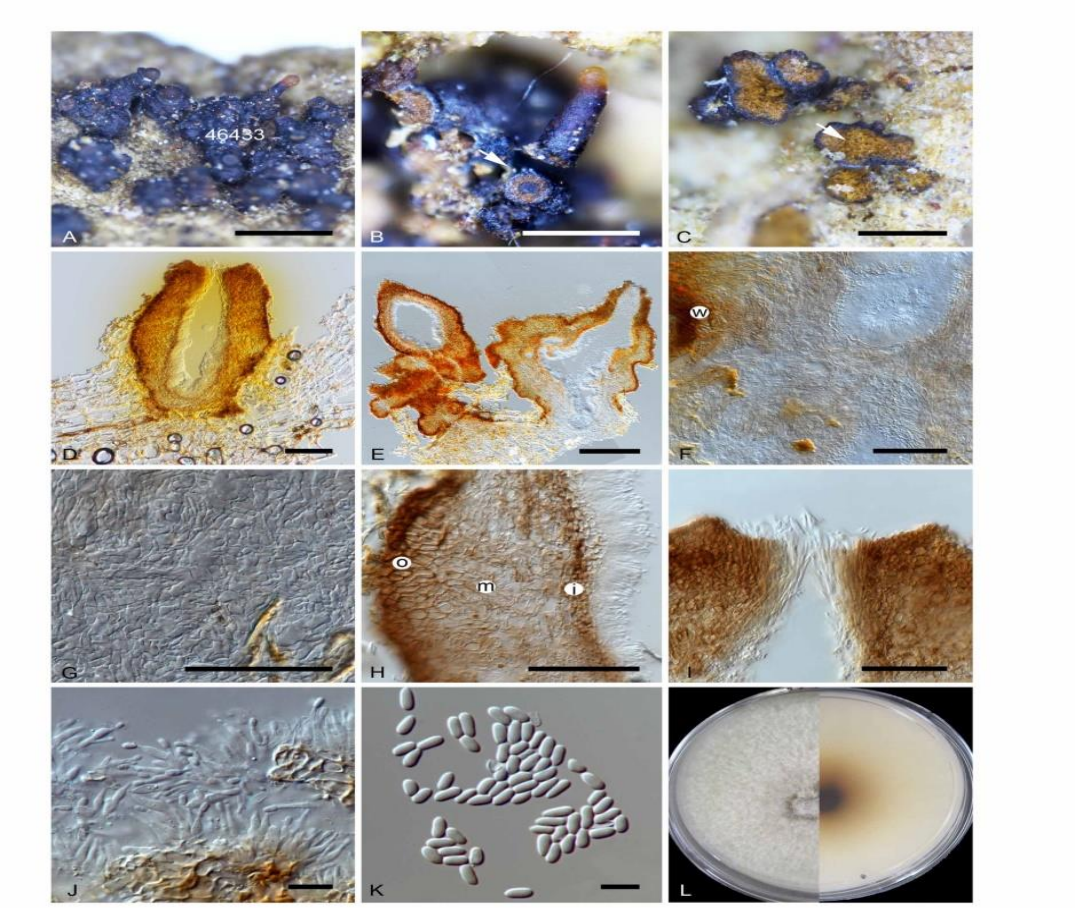

Fig. 9. Micrographs of *Myrtonectria myrtacearum* (holotype PREM xxxxx, ex-holotype CMW46433=PPRI xxxx). A. Conidiomata on the substrate. B, C. Broken neck and conidioma showing orange stromatic structure (arrows) and dark-coloured outer wall. D. E. Vertical section of conidiomata mounted in 85% lactic acid (D) exudating yellow to orange pigment. F, G. Pseudostromatic structure at the base of conidioma (w=conidiomatal wall). H. Close-up of conidiomatal wall and stromatic structure; in the middle *textura intricata* (m), and in the outermost (o) and the innermost wall (i). *texutura globosa* to *textura angularis*. I. Periphyses near the ostiole. J. Conidiophores and conidiogenous cells. K. Conidia. L. Culture grown at 25°C in the dark for 7 d on 2% MEA (right: below, left: above). Scale bars: A–C, E=250 µm; D=100 µm; F–I==50 µm; J=10 µm; K=5 µm.

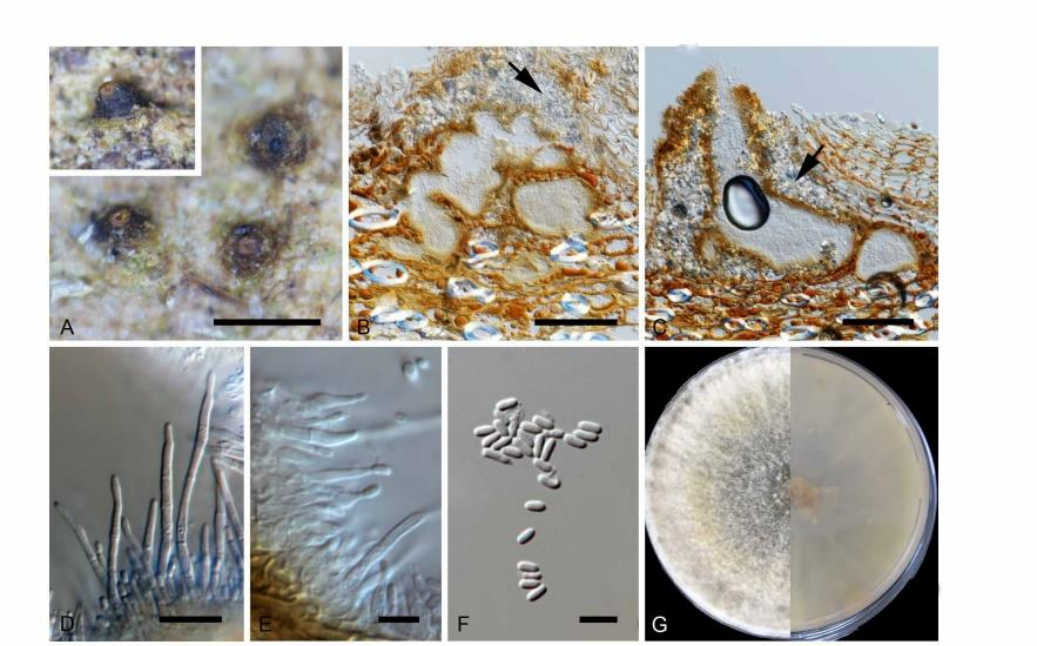

Fig. 10: Micrographs of *Celoporthe tibouchinae* (holotype PREM xxxxx, ex-holotype CMW44126=PPRI xxxxx) A. Conidiomata in the substrate. B, C. Vertical section of conidiomata showing shiny granular (arrows) in the middle layer of stroma. D. Paraphyses. E. Conidiogenous cells. F. Conidia. G. Colony grown at 30°C in the dark for 8 d on 2% MEA (left: above, right: below). Scale bars: A=250 µm; B, C=100 µm; D=10 µm; E, F=5 µm.

Fig. 11: Micrographs of *Celoporthe borbonica*. A. Ascostroma in the substrate. B. Vertical section of ascostroma. C. Close-up of ascostroma (sw=stromatal wall, p=prosenchymatous tissue, aw=ascomatal wall). D. Stromatic tissue in the middle. E. Vertical section of ascomatal neck. F–H. Ascus. I. Ascospores. J. Conidioma in the substrate. K. Vertical section of conidioma. L. Stromatic tissue of conidioma. M. Paraphyses. N. Conidiogenous cells. O. Conidia. P. Culture grown at 25°C in the dark for 7 d on 2% MEA. Scale bars: K=250 µm; A, B, J=100 µm, C=50 µm; D, E, L=25 µm; F–I, M–N=5 µm.

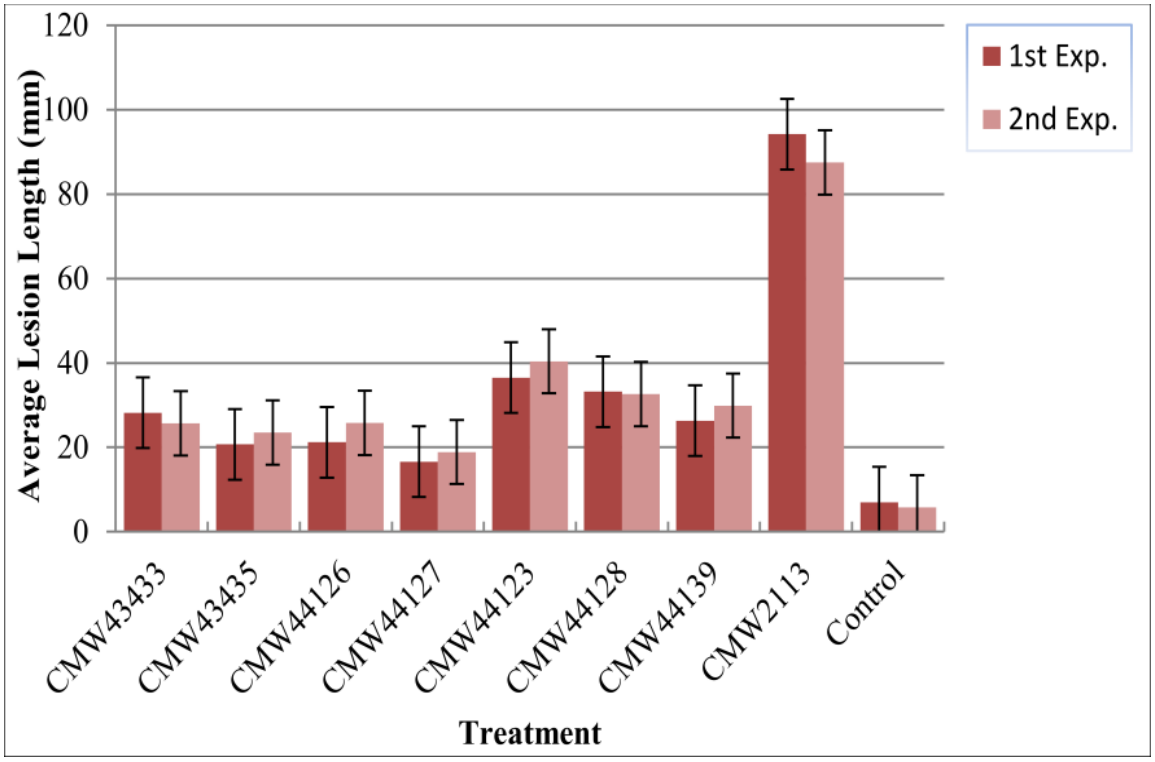

Fig. 12: Column chart showing the average lesion lengths resulting from inoculation trials for experiment 1 and 2 on stems of *Eucalyptus* clone TAG 5. The treatment includes two isolates of *C. myrtacerum*, two isolates of *C. tibouchinae* and two isolates of *C. borbonica* and a positive and a negative control. Vertical bars represent standard error of means.

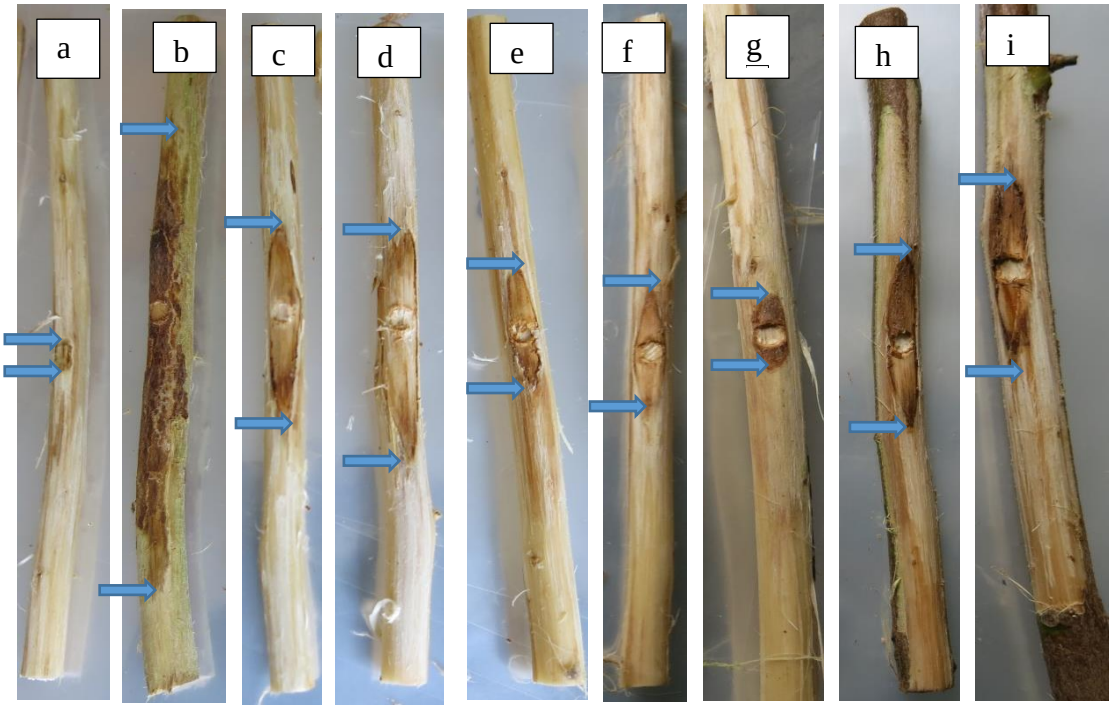

Fig. 13: Lesions resulting from inoculation of *Myrtonectria myrtacearum*, *Celoporthe borbonica* and *C. tibouchinae* onto *Eucalyptus* TAG 5 branches. (a, b) wound response respectively on the negative control and positive control CMW2113. Negative control showing absence of lesion development. Lesion associated with isolate CMW44123 (c), lesion associated with isolate CMW44128 (d), lesion associated with isolate CMW44139 (e), lesion associated with isolate CMW44126 (f), lesion associated with isolate CMW44127 (g), lesion associated with isolate CMW46433 (h), lesion associated with isolate CMW46435 (i). Arrows indicate the terminal ends of wound response.

SUMMARY

For many decades, fungi in the Cryphonectriaceae have caused serious stem canker diseases on woody plants worldwide. In the southern hemisphere, these fungi threaten *Eucalyptus* trees grown in plantations causing serious economic losses. The number of genera and species of Cryphonectriaceae has increased recently owing to the changes in taxonomic tools used to define fungal species. The review presented in this thesis highlighted topics relating to the Cryphonectriaceae including their morphology, taxonomy, origin, host jumps and shifts, species delineation as well as threats posed by virulent species. The number of genera and species of Cryphonectriaceae particularly from plants in the Myrtales remain under-studied, and their taxonomy has not been fully investigated. This justified an investigation of cankers on native Myrtales from La Réunion and South Africa for these fungi. The isolated species were characterized using morphology and a phylogenetic species concept, and their pathogenicity to *Eucalyptus* was determined. This resulted in the discovery of a previously undescribed genus and species in the Cryphonectriaceae causing stem disease from native *Heteropyxis natalensis* and *Syzygium cordatum* respectively in La Réunion and South Africa. The pathogenicity study showed that the new genus and species were mildly pathogenic to *Eucalyptus*. Description of the new taxa has added to the overall number of known genera and species in the Cryphonectriaceae. The work has also contributed knowledge regarding the pathogenicity of these fungi.