DECLARATION

I, **Angelica Marsberg** declare that the thesis, which I hereby submit for the degree **Magister Scientiae** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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CHAPTER 3

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PREFACE

Fungal endophytes are capable of infecting their host without any visible signs of disease. These diverse organisms have been isolated from all plants studied to date. Some species have undergone co-evolution with their host plants and offer benefits to their hosts. Others are often seen to be saprophytes or latent pathogens whose effects become noticeable when the host is stressed or dying. In South Africa, very few studies have been done on endophytes, an exception being the endophytic canker forming Botryosphaeriaceae pathogens.

Syzygium cordatum is a native South African tree that occurs along the East Coast of KwaZulu Natal. No studies have yet investigated the fungal diversity that may be associated with *S. cordatum*, unlike *Eucalyptus* trees which are well-studied. The aim of this study will be to determine the endophytic diversity of *S. cordatum* and to determine if the pathogenic fungi that occur on *Eucalyptus* trees are also associated with *S. cordatum*. This becomes problematic when a pathogen is introduced into a new environment and adapts to infect a new host with potentially damaging consequences to economically important plantations and natural ecosystems alike.

Chapter 1 of this thesis focuses on the literature available on fungal endophytes. The specific aim of the review was to gauge the complexity of these interactions and to try and understand the importance of these organisms in their environment, endophyte-host interactions, modes of infection, and the influence that endophytes have on their hosts as well as the co-evolution of life-history traits.

In **Chapter 2**, the diversity of fungal endophytes present in *S. cordatum* will be assessed. Two complimentary methods, namely isolate-based barcoding and environmental barcoding, will be used in combination to assess the diversity of fungal endophytes present. With culture-based methods, many fungal species may be missed as they do not grow in culture or grow so slowly that other fast growing species outgrow them. Culture-independent methods such as environmental barcoding make it possible to characterise multiple samples and species directly from the environment, thus increasing the probability that a greater diversity of endophytes will be detected. The latter technique is largely driven by next generation sequencing techniques such as 454 GS FLX Titanium pyrosequencing.

Chapter 3 aims to properly define a group of endophytes that belong to the Cladosporiaceae, Mycosphaerellaceae and Teratosphaeriaceae using multi-locus identification. Endophytes that were identified as belonging to the Cladosporiaceae, Mycosphaerellaceae and Teratosphaeriaceae in Chapter 2, and in a similar study done on a *Eucalyptus grandis* x *E. camaldulensis* GC540 hybrid clone, were subjected to multi-locus sequence analysis in order to establish their identities. These fungi are well-known leaf and shoot pathogens of *Eucalyptus* species and collectively cause the disease known as Mycosphaerella leaf blotch.

The endophytic fungi that will be identified in this study are expected to be native as they were isolated from a native South African tree. As *S. cordatum* and *Eucalyptus* species have a tendency to occur in the same environments, one will also expect that some fungal species will occur on both hosts. This study is expected to provide new insight into the potential diversity of fungi in southern Africa.

Factors affecting endophyte-host interactions

Abstract

Endophytes are organisms that inhabit plant organs and colonise plant tissues without causing obvious signs of disease. Much research has been done to elucidate the effects that endophytes have on their hosts, but the majority of these studies have been done on grass species that are considered model organisms. Evidence shows that endophytes evolved from closely related pathogenic fungi, because many endophytes are latent pathogens or saprobes that only sporulate when their hosts are stressed or dying. Hence, it is important to understand the principles surrounding endophyte biology and the effects that they could have on their hosts. There are specialised mechanisms involved enabling endophyte-host interactions to exist and these have co-evolved over millions of years. Endophytic symbionts affect the growth and fitness of their hosts, as well as their ability to tolerate biotic and abiotic stressors. In most cases, however, the ecological role and basis for the interaction between plants and their diverse assemblage of endophytes remain obscure and this is especially true for trees. Given the prominent presence of endophytes and the large part of biological diversity that they represent, further work to clarify these roles is urgently needed. This review aims to establish an understanding of the theory surrounding fungal endophytes, endophyte-host interactions, modes of infection, the influence of endophytes on their hosts, the co-evolution of life-history traits and why endophytes are important in their environments.

1. Introduction: Old term, new concept

Traditionally, endophytes were defined as organisms living within another organism, which was contrasted with epiphytes that live on the surfaces of their hosts (Kirk *et al.* 2008). Petrini (1991) suggested that endophytes are *"All organisms inhabiting plant organs that at some time in their life, can colonise internal plant tissues without causing apparent harm to the host"*. Despite the term "endophyte" prevailing over time, Wennström (1994) and Wilson (1995) argued, separately, that the term "endophyte" is no longer sufficient to describe the organisms that fit into this category.

Wennström (1994) argued that using only a single term is not adequate because "so-called endophytes" have few features in common. For instance, some species live asymptomatically within the plants, some may be systemic, while others are not, some endophytes remain mutualistic, while others become parasites. Wennström (1994) further pointed out that available data made it difficult to distinguish the "so-called endophytes" from other fungi and suggested that the term should be redefined to reflect the effect of the particular fungus. Wilson (1995) argued that the term "endophyte" described the type of association that a particular fungus or bacterium has with its host plant. Therefore, the term "endophyte" has changed from its original definition indicating location in a plant, to describing the fungus's association with the host. An important feature of "so-called endophytes" is that they produce no symptoms of disease on their hosts at a particular point in time. Wilson (1995), therefore, argued against Wennström's (1994) suggestion that "endophytes" have no features in common, as they are all found within their hosts' tissues asymptomatically.

"Endophytes" are often implied to be mutualists, but care should be taken when classifying them as such. This is because the association between certain endophytes and their host(s) can be dependent on environmental conditions and the different stages in their life-cycles, changing from mutualistic to pathogenic (Wilson 1995). Therefore, defining a species as an endophyte should take both definitions into account and Wilson (1995) thus suggested the following definition *"Endophytes are fungi or bacteria which, for all or parts of their life-cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease"*. This is similar to the original definition proposed by Petrini (1991).

All plant species studied to date harbour endophytes in their photosynthetic tissues that are taxonomically diverse and are comprised mainly of the ascomycetes (Arnold 2007; Arnold and Lutzoni 2007). Even though endophytic studies on trees, shrubs and ferns have shown that numerous endophytes are found in association with them, the majority of endophytic studies have focussed on the endophytes of grasses (Saikkonen *et al.* 1998). The probable reasons for this are because grass species are agriculturally important and they also serve as model organisms frequently used in ecological studies (Saikkonen *et al.* 1998; Arnold and Lutzoni 2007). There is, however, a clear recognition that the diversity of those endophytes associated with grasses, and less so than tree hosts, are nowhere near being fully circumscribed or understood.

Arnold (2007) highlighted the fact that it is important to understand the evolution and ecological roles that endophytes play in their respective hosts and environments. This is important, because evidence shows that endophytes evolved from plant pathogens that have adapted to an endophytic lifestyle and specialised mechanisms have evolved that make the symbiotic relationships possible (Clay 1988; Arnold 2007). Past and present research has

focused on investigating the symbiosis between endophytes and their hosts, how the endophytes colonise their hosts, the discovery and characterisation of metabolic products produced by these endophytes and how these endophytes evolved along with their hosts (Arnold 2007). Given that the diversity of endophytes is vastly understudied, it is clear that we are far from fully understanding the ecology and evolution of this important group of organisms.

This review aims to establish a basic knowledge of endophytes and lays a foundation towards understanding the complex patterns that will emerge from characterising their diversity in a native host and environment. The review focuses on endophyte-host interactions, the modes of infection, the influence on and composition of endophytes in their hosts and the co-evolution of life-history traits.

2. The evolution of endophyte – host interactions

Endophytic fungi have various types of relationships with their hosts, which can be broadly classified as mutualistic, saprophytic or parasitic in the form of latent pathogens (Freeman and Rodriguez 1993). Mutualism involves the exchange of benefits and costs equally between host and endophyte (Schardl *et al.* 2004). Examples of such fungi are those that aid in setting up a defence against pests and pathogens, a phenomenon known as acquired plant defences (Cheplick and Clay 1988). Endophytes of woody plants may also aid plant defences through the production of alkaloids that prevent invasion by other pathogens, as well as deterring insect herbivores (Carroll 1988).

Latent pathogens are endophytes that become pathogenic when their host is stressed, such as during a drought (Carroll 1988; Schulz and Boyle 2005), whilst saprophytes sporulate only

once the host plant or tissue has undergone senescence (Kehr 1991; Schulz *et al.* 1999). Because latent pathogens and saprophytes can establish long-term relationships with certain host plants, it is clear that these fungi are adapted to an endophytic life-style as their hosts have no visible disease symptoms upon infection (Mostert *et al.* 2000; Schulz and Boyle 2005). For example, the presence of the fungal pathogen, *Deightoniella torulosa*, in symptomless leaves of *Musa acuminata* confirms that some fungal pathogens have a latent endophytic stage before disease symptoms develop (Photita *et al.* 2004). This same fungus has also been shown to be a saprophyte on the dead leaves of *M. acuminata* (Photita *et al.* 2003; Photita *et al.* 2004).

Vertical and horizontal transmission represent two possible mechanisms by which fungal endophytes are transmitted (Figure 1). In the case of horizontal transmission, endophytes are transmitted via sexual or asexual spores to infect typically above ground parts of new plants (Carroll 1988; Clay and Schardl 2002; Saikkonen *et al.* 2004; Rodriguez *et al.* 2009b). Where vertical transmission is involved, endophytes are transmitted from the plant to its offspring via the seeds (Carroll 1988; Clay and Schardl 2002; Saikkonen *et al.* 2004; Rodriguez *et al.* 2009b). In woody plants, horizontal transmission is dominant, whilst vertical transmission is important in grasses (Clay and Schardl 2002; Saikkonen *et al.* 2004). Mode of transmission appears to affect the degree to which endophytes are mutualistic with their hosts (Saikkonen *et al.* 1998). Vertically transmitted endophytes tend to evolve a mutualistic relationship with their hosts, as reproduction and fitness of both the endophyte and plant are closely linked (Ewald 1987; Lipsitch *et al.* 1996; Saikkonen *et al.* 1998). Endophytes that are transmitted horizontally are less likely to have a mutualistic relationship with their hosts as reproduction of the host and endophyte is not linked (Ewald 1987; Lipsitch *et al.* 1996; Saikkonen *et al.* 1998).

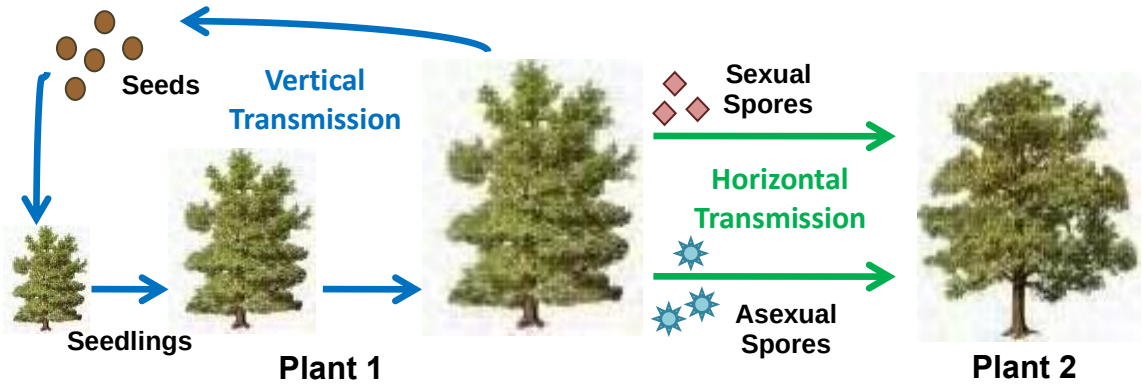

Figure 1: Vertical and horizontal transmission of endophytes and their life-cycles within and between hosts (From Saikkonen *et al*. 2004)

The numerous endophytic fungi associated with an individual host or diverse range of hosts suggests that the relationship between plants (particularly grasses) and endophytes must be ancient (Clay 1993; Strobel 2003; Zhang *et al.* 2006; Krings *et al.* 2007). Taxonomic evidence shows that endophytes evolved from pathogenic fungi associated with plants, as endophytes are closely related to pathogens found on identical or closely related hosts (Carroll 1988; Clay 1988; Saikkonen *et al.* 1998). In grass species, it remains unclear how this particular group of endophytes, residing in Claviciptaceae, became endophytes. One hypothesis is that the Claviciptaceae are ancestors of fungi in *Balansia*, a parasitic fungus (Clay 1993). Alternatively, the Claviciptaceae evolved from a systemic, non-pathogenic *Balansia*-like fungus (Clay 1993).

The underlying molecular basis for the endophytic life-style remains unclear. Using approaches whereby genes are disrupted resulting in the switch from pathogen to mutualist, provides insight into the molecular mechanisms that bring about pathogenicity and mutualism (Freeman and Rodriguez 1993; Eaton *et al.* 2010b). Freeman and Rodriguez (1993) found that a single mutation at a single locus of path-1 *Colletotrichum magna* mutants could change an isolate from being pathogenic to non-pathogenic. In contrast, Tanaka *et al*. (2006) isolated

mutants of *Epichloë festucae* that were unable to establish mutualistic relationships with their host plant. They found a mutated gene coding for NoxA, a subunit of the multi-subunit NADPH oxidase complex. Plants infected with the *NoxA* mutant showed severe stunting and subsequent death (Tanaka *et al.* 2006). Two additional components of the Nox complex in *Epichloë festucae* were also found to be important in the maintenance of mutualism with their host, namely NoxR and RacA (Takemoto *et al.* 2006; Tanaka *et al.* 2008). This indicates that the Nox complex is closely linked to the pathogenicity and maintenance of mutualism in endophytes (Eaton *et al.* 2010b). Iron siderphores and the stress activated MAP kinases are also involved in the maintenance of the mutualistic relationships between host and endophyte (Johnson 2008; Eaton *et al.* 2010a). From these studies, it is evident that while a single mutation can break down the mutualism between host and endophyte, more than one gene is involved in this maintenance, even though a single mutation in a pathway can over-ride the balanced system.

3. Direct effects of endophytes on their hosts

Endophytes have evolved together with their hosts over a long period of time (Strobel 2003; Pimentel *et al.* 2010). For this reason, one would expect to see a form of a "modified arms race" taking place, in which the endophyte, for example, produces compounds closely resembling those of the host (Goodman *et al.* 1986). It is also known that endophytes have an effect on the growth of plants, their fitness and stress tolerance (Clay 1990; Rodriguez *et al.* 2009a; Rodriguez *et al.* 2009b). Likewise, it has been shown that endophyte-infected plants have a greater fitness advantage when compared to uninfected plants (Clay 1990; Rodriguez *et al.* 2009a; Rodriguez *et al.* 2009b). Furthermore, endophytes have developed

genetic mechanisms to transfer information between themselves and their host plants (Strobel 2003; Pimentel *et al.* 2010).

The biochemical mechanisms by which endophytes affect the growth of plants are poorly understood (Cheplick and Cho 2003; Schardl *et al.* 2004; Spiering *et al.* 2006). Endophytes might influence plant growth by producing plant hormones as well as glycosidases and proteases that affect the metabolic pathways of the plant, potentially influencing photosynthesis, stomatal conductance and water regulation (Goodman *et al.* 1986; Spiering *et al.* 2006). Phytohormones, such as indoleacetic acid, produced by an endophyte may also induce slight changes in plant growth (Schardl *et al.* 2004).

The toxic effect that endophyte-infected grasses have on livestock has long been observed, and therefore, it seems that endophytes have an effect on mammalian herbivory (Clay 1988). This toxicity is due to the production of alkaloids by the endophytes, as uninfected plants lack these alkaloids (Waller *et al.* 2005). The typical bitter taste that is associated with alkaloids would deter herbivores from consuming infected plants (Schardl and Phillips 1997). It has also been observed that the production of alkaloids affects insect herbivores (Johnson *et al.* 1985). For example, elm bark beetles attack the bark of elm trees, which allows the endophyte *Phomopsis oblonga*, to invade (Claydon *et al.* 1985). The subsequent compounds produced by the endophyte deter the beetles from breeding in the trees and have been shown to reduce the population size and damage to the trees (Claydon *et al.* 1985; Carroll 1988).

Certain classes of endophytes are known to affect the ability of plants to handle abiotic stresses, such as drought (Bae *et al.* 2009). Malinowski and Belesky (2000) stated that there are several mechanisms that are used by cool-season grasses to adapt to conditions of

drought, and these include morphological and physiological changes. Firstly, upon endophyte infection, there is an increase in root dry matter, which improves water absorption. Secondly, endophytes may aid the plant in rapid stomata closing, aiding to reduce water loss through transpiration, but the mechanism by which this occurs is not known. Thirdly, there is increased water storage in endophyte infected plants. This may be due to the accumulation of solutes in the endophyte infected tissues (Malinowski and Belesky 2000) as observed in a study conducted on *Theobroma cacao* seedlings infected with endophytes (Bae *et al.* 2009). The seedlings had a delayed response to drought and there were changes in the seedlings physiology, with advanced root growth and increased water storage (Bae *et al.* 2009). These changes in physiology may have been due to chemical signals released by the endophyte (Malinowski and Belesky 2000). It, therefore, appears that grass and some non-grass species undergo the same adaptive mechanisms involving endophytes during periods of drought, and it would be interesting to see if more non-grass species have similar adaptations.

The presence of endophytes can prevent or reduce the severity of pathogen infections. *Lophodermium conigenum* is an endophyte of Scots pine needles that sporulates only after the death of the pine needles. It appears to prevent the invasion of *L. seditiosum*, a pathogen on young trees, which is only able to colonise the host when *L. conigenum* is absent (Minter 1981; Carroll 1988). In woody angiosperms, it was found that endophyte infection reduced the amount of damage to leaves and the loss thereof when *T. cacao* was infected with a foliar pathogen, a species of *Phytophtora* (Arnold *et al.* 2003). It was shown that the inoculation of leaves with endophytes isolated from infected asymptomatic hosts, decreased leaf necrosis as well as leaf mortality. This was localised to only those tissues infected with endophytes (Arnold *et al.* 2003). Ganley *et al*. (2008) further demonstrated that fungal endophytes

associated with *Pinus monticola* were also effective at increasing the survival of their hosts when they were infected with *Cronartium ribicola*, the pathogen that causes white pine blister rust. However, only a few such examples exist and it is still unclear if one or more of these endophytes prevent pathogens from invading their host.

Despite the numerous potential benefits endophytes confer on their hosts, they also incur costs. It has been suggested that endophytes associated with the grass species *Lolium perenne*, for example, are costly and reduce the growth rates of their host when soil nutrition is low or after a drought (Cheplick 2007). It, therefore, appears that when resources are limited, there may be a "metabolic cost" to the endophyte infected host because of the limited supply of resources and photosynthetic products. Cheplick (2007) found that under stressful, low resource conditions endophyte infected plants had a reduction in the root:shoot ratio. As root growth depends on photosynthetic products transported into them and endophytes consume unknown amounts of these products, it is clear that the endophyte-host interaction is not beneficial during unfavourable conditions (Cheplick 2007).

Latent pathogens infecting their hosts and that live asymptomatically as endophytes for a period of their life cycle represent an example of how some endophytes negatively affect their hosts (Sakalidis *et al.* 2010). If the host is subjected to stressful conditions, the pathogen cycle may be activated and disease occurs. A good example of this life strategy is found with the Botryosphaeriaceae, which are well-known latent pathogens responsible for canker formation and dieback particularly on trees (Sangchote 1991; Slippers and Wingfield 2007; Sakalidis *et al.* 2010). The risk that latent pathogens pose is still largely unknown as they infect their host asymptomatically and only become pathogenic when the host is stressed (Sakalidis *et al.* 2010). Further research is, therefore, required to understand the cues that lead to pathogenicity and the risks these endophytes may have once the switch occurs.

4. Complexity of the endophyte infection process

Endophytes produce the enzymes necessary for penetration and infection of their hosts and their growth can be localised and intercellular in above-ground tissues (Boyle *et al.* 2001; Schulz *et al.* 2002). A study was done to compare the infection process of endophytes and pathogens and it was found that endophytes penetrate their host via the stomata and along the anticlinal walls of the epidermal cells, whilst the pathogen penetrated the cell wall directly (Boyle *et al.* 2001).

As endophytes and pathogens infect their hosts by penetration, plant defence mechanisms may influence endophyte colonisation as the host recognises the endophyte as non-self as if it were a pathogen (Kogel *et al.* 2006). Penetration by the endophyte is followed by the release of elicitor-active molecules by the host (Kogel *et al.* 2006). In order for the endophyte to be accommodated, the host plant must recognise it as “friendly”. This recognition occurs via the hosts’ receptor-kinase-mediated transmembrane signalling pathway (Chapela *et al.* 1991; Stracke *et al.* 2002; Kogel *et al.* 2006). This same pathway also recognises pathogens non-specifically, which suggests that a common characteristic may have developed during the evolution of symbiotic relationships for the similar recognition of endophytes and pathogens by plants (Akiyama *et al.* 2005; Kogel *et al.* 2006). Plant defences are active against both the plant pathogen and endophyte, but the plant pathogen is able to suppress these reactions that lead to the onset of disease, whilst the endophyte can tolerate these reactions (Schulz *et al.* 1999; Schulz *et al.* 2002).

It is unknown to what extent endophytes by-pass the plants’ defence system in order to be recognised as “friendly”, as many pathways are involved in plant defence responses and these all need to be in balance in order to maintain the endophyte-host interactions (Schulz *et*

al. 2002; Kogel *et al.* 2006). Mutualistic relationships result from a balance in the combination of the interactions between the internal and external environments, physiology of both the host and endophyte as well as their genetic make-up, whereas parasitism is unbalanced (Schulz *et al.* 2002; Kogel *et al.* 2006). These interactions, particularly the molecular mechanisms involved, are yet to be understood. Therefore, further molecular studies are required to understand the various physiological mechanisms and endophyte-host interactions involved during host plant colonisation.

5. Factors affecting endophyte communities on host plants

Because endophytes have evolved along with their hosts, some may have developed a degree of host affinity and are thought to be host-specific (Zhou and Hyde 2001). Cannon and Simmons (2002) set out to evaluate the hypothesis that endophyte communities are host-specific in tropical environments. However, no prominent fungal groups were identified for particular plant species, which implied that the degree of host-specificity was low. Furthermore, Arnold *et al*. (2003) found that host-specific leaf chemistry influenced endophytic growth in culture. Endophytes were isolated from *Theobroma cacao* (Arnold et al. 2003), *Heisteria concinna* (Arnold *et al.* 2000) and *Ouratea lucens* (Arnold *et al.* 2000) and subsequently grown on media containing leaf extracts from the three tree species. The highest level of growth was observed for those endophytes grown on the media containing the leaf extracts from which they were originally isolated from (Arnold *et al.* 2003). It was concluded that leaf chemistry may affect the competition between endophytes *in planta* by controlling their growth and influencing species composition and apparent host affinity of these species (Arnold *et al.* 2003).

Fungal diversity may in the past have been underestimated due to the bias imposed by culturing (Cannon and Simmons 2002). Those endophytes that grow more rapidly in culture, outgrow and out compete slow growing strains, and this gives the impression that a particular fungal group is dominant in a specific host (Cannon and Simmons 2002). Little is known about the diversity and abundance of those endophytes that do not grow well or do not grow at all in culture (Arnold *et al.* 2007). However, techniques based on environmental PCR on surface sterilised leaves may aid in determining the diversity and host range of those endophytes lost during the culturing process (Arnold *et al.* 2007). A study on the diversity of the endophytes of *Pinus taeda* (Arnold *et al.* 2007), for example, indicated that with the application of environmental PCR, more basidiomycetous endophytes were recovered than through culturing alone.

Non-native plants appear to be more diverse in their endophyte assemblages (Fisher *et al.* 1993; Fisher *et al.* 1994; Taylor *et al.* 1999; Hoffman and Arnold 2008; Shipunov *et al.* 2008). Hoffman and Arnold (2008) compared the endophytes of three closely related conifer tree species, including two native species in their native range and a non-native *Platycladus* species. Greater numbers of endophytes were found to be associated with the non-native *Platycladus* sp. than with the native hosts. The *Platycladus* sp. also had more opportunistic "generalist" endophytes associated with it than the native hosts. Most of the endophytes isolated were Dothideomycetes, while Sordariomyctes, Eurotiomycetes and Pezizomycetes were isolated to a lesser extent. Species of *Phoma*, *Aureobasidium*, *Alternaria*, *Cladosporium* and *Xylaria* were found to be common on the non-native *Platycladus* sp. than on the native species (Hoffman and Arnold 2008). This same trend was observed by Shipunov *et al*. (2008). The dominantly isolated endophytes were species of *Alternaria* and *Cladosporium*, as well as species of *Botrytis* and *Fusarium* (Shipunov et al. 2008).

The Endophyte Enemy Release theory may explain the above-mentioned phenomenon of increased diversity in non-native plants (Keane and Crawley 2002; Evans 2008). The balanced relationship between the endophyte and host is lost when plants are moved to new, exotic environments as there is an incomplete association of both co-evolved endophytes and natural enemies. Naturally, co-evolved endophytes protect their hosts from invasion by other fungal species, therefore, the absence of these endophytes make the exotic plants highly susceptible to fungal invasion, thus increasing the endophytic diversity in these plants (Keane and Crawley 2002; Evans 2008). However, the opposite may also hold true where non-native plants are less diverse than native plants. This may be due to the resistance conferred by the non-native plants' natural endophytes, which protect the plant from being invaded by the endophytes found in the new, exotic environment (Mitchell and Power 2003; Levine *et al.* 2004). This phenomenon is known as the Biotic Release Hypothesis (Mitchell and Power 2003; Levine *et al.* 2004).

A number of studies have investigated the effect of season on fungal endophyte diversity (Collado *et al.* 1999; Arnold and Lutzoni 2007; Göre and Bucak 2007; Guo *et al.* 2008). Rainfall gradient affects endophytic diversity and the greatest endophyte density is observed during the wet seasons as this promotes the dispersion of the fungal spores leading to an increase in host plant colonisation (Collado *et al.* 1999; Suryanarayanan *et al.* 2002; Göre and Bucak 2007; Guo *et al.* 2008). Differences in the variety of endophytic species isolated also differed between seasons (Collado *et al.* 1999; Göre and Bucak 2007). Guo *et al*. (2008) found that the lowest number of endophytes were isolated from *Pinus tabulaeformis* in summer. In summer the pine needles and twigs were newly grown and endophytes had not yet had the chance to establish themselves (Guo *et al.* 2008).

Leaf age appears to affect endophyte diversity on a given host (Suryanarayanan *et al.* 1998; Arnold and Herre 2003; Seena and Sridhar 2004; Pang *et al.* 2008). Arnold and Herre (2003) conducted a study on tropical fungal endophytes of *T. cacao* and considered the effect of canopy cover and leaf age on endophytic diversity. Young, unopened leaves harboured fewer endophytes than mature, older leaves (Rodrigues 1994; Arnold and Herre 2003). The reasoning was that the leaves accumulated aerial spores from the canopy cover throughout their life-time, unlike those found in the clearings. The endophytic diversity between covered and uncovered plants, however, equilibrated over time as endophytes may prevent infection from other fungi (Arnold and Herre 2003). This supports the hypothesis that horizontal transmission of endophytes occurs in woody plants, as older plants and tissues accumulate more endophytes than younger tissues (Arnold and Herre 2003; Guo *et al.* 2008). Studies done on mangrove trees have also observed that mature leaves have a greater endophytic diversity than younger ones and this phenomenon has also been observed in grasses that are known to have vertically transmitted endophytes (Clay 1988; Seena and Sridhar 2004; Pang *et al.* 2008).

6. Conclusions

Fungal endophytes constitute a diverse group of organisms that exist asymptomatically within their hosts and influence plant fitness and growth. Thus far, endophytes have been found to be associated with all plants studied, however, the extent of their diversity, host range and distribution remains poorly defined. Successful colonisation of the host relies on an interplay between the abundance of fungal spores, habitat, mode of transmission, season and the susceptibility of the host plant to infection (Arnold and Herre 2003). Specialised mechanisms are involved that make these endophyte-host interactions possible and have evolved over

millions of years to allow for the successful establishment of the symbiotic relationships. Understanding these evolutionary mechanisms is a daunting prospect, as the numerous factors that influence, or have influenced, these interactions need to be teased apart.

It is not easy to establish why a particular endophyte is found associated in a specific host. One can ask whether finding an endophyte associated with a particular plant might imply that it is specific to that plant, a coincidental infection, or whether it is a result of under sampling of other hosts. Some studies have shown that certain endophytes favour particular hosts, yet there are other studies contradicting this view. Therefore, it is important that more studies are conducted over a range of hosts and tissues in order to elucidate how and why plants and endophytes interact, specifically or non-specifically, and also to determine if a particular endophyte evolved exclusively with its host plant. Given the complexity of endophyte-host interactions, it would be useful not to make these types of studies inordinately broad, but to focus on a specific endophytic system that can be investigated further.

Certain endophytes with broad host ranges may not be mutualistic with all the hosts with which they are associated and they might adversely affect some of their hosts. There is a fine line between an endophyte being a mutualist or being a pathogen and this is underpinned by the fact that some endophytes have evolved from pathogenic fungi and both invade their host plant to obtain some form of benefit (Saikkonen *et al.* 2004). Mutualism is the reciprocal of parasitism and interactions may be positive or negative depending on the circumstances which depend on the host, environment and the genotypes of those organisms involved (Egger and Hibbett 2004; Sachs and Simms 2006).

It is clear that the knowledge pertaining to the diversity, ecology and evolution of endophytes, particularly those associated with woody plants, is far from complete. This is despite the

ubiquity, diversity and prominence of these organisms in the environment that also supports a relevant ecological role. There, however, seems little opportunity to define these organisms more accurately before a deeper understanding is established regarding how they interact with their hosts. Evaluation of host diversity, range, colonisation and the transmission of endophytes, in particular, is required to build a firm foundation to understand the ecological role of these organisms.

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Environmental barcoding of the endophytic fungi associated with native *Syzygium cordatum* in South Africa

Abstract

Endophytes represent a rich source of biodiversity. Some species are latent pathogens of plants, which can have important implications for tree health and quarantine. The biodiversity of the endophytic fungi associated with healthy *Syzygium cordatum* trees was studied using isolate-based barcoding and culture-independent environmental barcoding using 454 GS FLX Titanium pyrosequenced mini-barcodes. This tree species was chosen because it is native to South Africa and closely related to commercially important *Eucalyptus*. Over 23 000 mini-barcodes were obtained from the environmental barcoding and these represented 15 orders, 20 families and 92 Molecular Operational Taxonomic Units (MOTU's). Species of Mycosphaerellaceae were the dominant group of endophytes detected with pyrosequencing, representing 13 different MOTU's, whilst species of *Microdochium* and *Cladosporium* also represented dominant clusters. Two hundred and fifty isolates were identified from a single tree using the full-length Internally Transcribed Spacer (ITS) region of the nuclear ribosomal RNA gene operon and these represented 13 orders, 22 families and 42 species. Approximately 12% of the isolates could not be satisfactorily identified. A species of *Pestalotiopsis* was the most dominant endophyte isolated, while others were *Bionectria ochroleuca*, *Colletotrichum* aff. *gloeosporioides* and *Neofusicoccum mediterraneum* based on BLAST results from the GenBank DNA sequence database. This first study on the endophytic diversity associated with *S. cordatum* showed that a significant number of fungi can be found in a single tree, and that some of these are associated with latent pathogen groups.

1. Introduction

In 2004, the International Barcode of Life (IBoL; http://www.ibol.org) was established to develop a rapid, global method for the identification of organisms based on DNA barcodes (von Cräutlein *et al.* 2011). DNA barcoding identifies organisms from short DNA sequences amplified from specified gene regions using universal primers (Hajibabaei *et al.* 2007; Virgilio *et al.* 2010). A proposal has been submitted to the Consortium for the Barcode of Life (CBOL; http://www.barcoding.si.edu/) for the approval for the use of the Internally Transcribed Spacer (ITS) nuclear ribosomal DNA region as the primary fungal marker (Schoch *et al.* 2012). Sequences are compared against databases representing barcodes from previously identified individuals, and genetic distance methods are used to interpret these results in order to identify the unknown organism (Hebert *et al.* 2003a; Hajibabaei *et al.* 2007; Seifert 2009; Goldstein and DeSalle 2010; Virgilio *et al.* 2010). The approach thus differs from conventional identification using DNA sequence comparisons with publically available databases such as the GenBank DNA sequence database (Seifert 2009). This is primarily because DNA barcoding constructs reference libraries in which the barcode sequences are linked to the original voucher specimen or DNA, which is stored for future analyses (Savolainen *et al.* 2005; Floyd *et al.* 2010). Ecological data are also linked to the barcodes that enable more powerful comparisons to be made in the future (Valentini *et al.* 2009; Damm *et al.* 2010; Heimeier *et al.* 2010).

Environmental barcoding involves the characterisation and identification of genetic material obtained directly from the environment, or of microbial communities obtained directly from other organisms (Riesenfeld *et al.* 2004; Tringe and Rubin 2005). This allows for the study of diverse communities from different niches where conventional or culture-based methods are inadequate to detect the incredibly numerous, poorly defined, specious groups and those species which are in low abundance or deemed unculturable (Riesenfeld *et al.* 2004; Tringe and Rubin 2005). Environmental barcoding is largely driven by next

generation sequencing technologies that are massive parallel sequencing techniques generating thousands of sequences in a single experiment (Hutchison 2007). This overcomes the limitations of traditional or culture-based methods to detect those species that are rare, those considered unculturable or where communities are complex (Hutchison 2007).

Environmental barcoding generates mini-barcodes that are shorter, incomplete fragments of the particular gene region used to form complete barcodes, without the need for assembly of sequence reads (Taylor and Harris 2012). These smaller fragments are also preferentially amplified from environmental samples where DNA degradation may be a problem (Bellemain *et al.* 2010). Mini-barcodes also include important differences to other molecular profiling techniques, such as Terminal Restriction Fragment Length Polymorphisms (T-RFLPs), Denaturing Gradient Gel Electrophoresis (DGGE) and microsatellites, which only give a profile of the community that can be compared to the profiles of others (Rosenzweig *et al.* 2012).

Mini-barcodes provide some level of identification for community members. In this regard, 454 GS FLX pyrosequencing has the greatest potential of the various next generation sequencing techniques for environmental barcoding, because it produces longer DNA sequences. Furthermore, improvements in the chemistry for 454 GS FLX Titanium pyrosequencing currently promises sequences of 500 base pairs in length (Huse *et al.* 2007; Creer *et al.* 2010; Gilles *et al.* 2011). This overcomes the limitations of other techniques also used for identification, such as the cloning of amplicons obtained from environmental samples. The latter approach can generate complete barcodes, but is time-consuming and expensive to generate if they were to fully cover the diversity of such environmental communities (Edwards *et al.* 2006; Harkins and Jarvie 2007).

In order to fully utilise the data generated by the mini-barcode approaches, it would be ideal to have vouchered databases against which to compare the mini-barcodes obtained (Ratnasingham and Hebert 2007; Hajibabaei *et al.* 2011; Shokralla *et al.* 2011; Rosenzweig *et al.* 2012). In the absence of such databases, mini-barcodes are searched against databases, such as the GenBank DNA sequence database, which are publically available. In the latter case, even identifying complete barcodes or fragments of the ITS region is usually troublesome, because of a large number of incorrectly identified or poor quality sequences in the GenBank DNA sequence database that often distort identifications (Nilsson *et al.* 2006; Seifert 2009). Complementation of an environmental approach with that of conventional methods or culturing, where these cultures are also barcoded, enables a more robust database to be created and against which the generated mini-barcodes can be compared. This approach would work best if such a dedicated barcode database is built systematically over time and only when it is as representative as possible (Hebert *et al.* 2003a; Hebert *et al.* 2003b; Hajibabaei *et al.* 2007; Seifert 2009; Goldstein and DeSalle 2010; Virgilio *et al.* 2010; Monchy *et al.* 2011).

Syzygium cordatum, commonly known as waterberry, is a South African native tree belonging to the Myrtaceae (Myrtales). It is evergreen, water loving and occurs widely on the East Coast of South Africa (Palgrave 1977). *Syzygium cordatum* resides in the same family as *Eucalyptus* trees, that were introduced into South Africa for use in commercial plantations (Poynton 1979). The focus of most studies that have investigated the pathogens of *S. cordatum* have, in the past, considered the overlap of pathogens between these tree species (Crous and Wingfield 1991; Slippers *et al.* 2005; Heath *et al.* 2006; Nakabonge *et al.* 2006a; Nakabonge *et al.* 2006b; Pavlic *et al.* 2007b; Heath *et al.* 2011). The value of such studies lies in the fact that co-infections are a threat to both plantations and natural ecosystems alike, especially because these trees are often found in close

proximity to plantations (Crous and Wingfield 1991; Slippers *et al.* 2005; Pavlic *et al.* 2007b).

Some pathogens of *Eucalyptus* and *S. cordatum* trees can occur as endophytes, which are fungi that occur asymptomatically within their hosts (Petrini 1991). An example is that of the well-known canker pathogens belonging to the Botryosphaeriaceae, which are known to be endophytes of *Eucalyptus* as well as of *S. cordatum* (Smith *et al.* 2001; Pavlic *et al.* 2007b; D. Pavlic *et al.* 2009; Slippers *et al.* 2009). However, the full complement of endophytes associated with *S. cordatum* has not been studied and the proportion of endophytes that are latent pathogens, which form part of the endophytic community, is unknown. For *Eucalyptus*, a limited number of studies have been undertaken (Fisher *et al.* 1993; Bettucci *et al.* 1999; Sánchez Márquez *et al.* 2010), and only recently a pilot study has been conducted in South Africa for *Eucalyptus grandis* x *E. camaldulensis* GC540 hybrid clones (Pillay 2012).

In this study, environmental barcoding using 454 GS FLX Titanium pyrosequencing of fungal DNA extracted directly from plant material was used to study the biodiversity of fungal endophytes associated with the leaves, twigs, branches and trunk increments of healthy *S. cordatum* trees. This was complemented by a culture-based approach where endophyte cultures were fully barcoded based on ITS sequences. This study serves as an important pilot study to determine the levels of infection and biodiversity of these endophytes to serve as a baseline for planning if more surveys are to be done in the future.

2. Materials and Methods

2.1.1. Sample collection

From each tree, four leaves each from four twigs from four branches located in different positions on the tree and one trunk increment with the bark attached, were collected from three healthy *Syzygium cordatum* trees, 10 m apart. Sampling was done in a private nature reserve in the Mtubatuba area of KwaZulu Natal, South Africa (E32'9"54.1; S28'29"53.0). Samples were transported to the laboratory for further processing. Ten disks (5 mm in diameter) were cut from each leaf, and five pieces (3 mm long) were taken from each twig and branch. These and all of the trunk increment samples were surface disinfected by first rinsing in 10 % hydrogen peroxide for 3 to 5 minutes, followed by two washes for one minute each with sterilised distilled water.

Samples were stored in Eppendorf tubes at - 40 °C for later use in the pyrosequencing. Double the number of plant pieces were taken from tree 1, with half stored in Eppendorf tubes for pyrosequencing and the other half plated for endophytic isolations onto 2 % Malt Extract Agar (MEA) (20 g malt extract, 20 g agar, 1L distilled water; Biolab, Midrand, South Africa). These were incubated at 25 °C. The endophytes that emerged were purified and incubated further at 25 °C. Cultures were checked continuously for 30 days to ensure that even slow growers, that were not excessively overgrown, were isolated. These cultures are maintained in the culture collection (CMW) at the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.

2.2. Environmental barcoding using 454 GS FLX Titanium pyrosequencing

2.2.1. DNA extractions

Total genomic DNA was extracted from the plant samples using the Qiagen DNeasy Plant Mini Kit as per manufacturer's instructions (Qiagen Sciences Inc, Germantown, MD). The

plant material stored in the Eppendorf tubes were freeze dried and ground with a mortar and pestle prior to extractions. PCR amplification was performed in two-steps. In the first PCR, the ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns 1993) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.* 1990) primer pair was used to amplify the Internally Transcribed Spacer (ITS) nuclear ribosomal DNA for each of the plant material samples. The volume for each reaction was 25 µl with 1 µl of genomic DNA, 15 pmoles of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 1x NH_4 Reaction Buffer (Roche Products Pty Ltd, South Africa), 1.2 mM $MgCl_2$ (Roche Products Pty Ltd, South Africa), 75 µM dNTPs (Roche Products Pty Ltd, South Africa) and 5 units of Fast Start Taq (Roche Products Pty Ltd, South Africa). The final volume of 25 µl was achieved with the addition of nuclease-free water. The cycle parameters were as follows: 94 ºC denaturation for 5 minutes, followed by 35 cycles of denaturation for 30 seconds at 94 ºC, annealing at 50 ºC for 30 seconds, and elongation at 72 ºC for 45 seconds. A final extension step followed at 72 ºC for 7 minutes. The DNA extracted from tree 1 inexplicably degraded even though all the plant material for the three trees was handled in the same manner. Therefore, these samples could not be amplified in order for the subsequent steps to be completed satisfactorily. These samples were discarded, resulting in the inclusion of only two trees for pyrosequencing analysis.

Gel electrophoresis validated the success of the PCR under ultraviolet (UV) light. Gel purification of each PCR product was done using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA) as per manufacturers' instructions. This was done in order to ensure that artefacts that were smaller or larger than the fragment of interest were not included in the subsequent steps. The concentration of each sample was determined using a NanoDrop™ 1000 Spectrophotometer version 3.7 (Thermo Fisher Scientific Inc. NYSE: TMO) and each sample was standardised to 5 ng/µl.

The gel purified samples were used as templates for the second round of PCR amplifications, whereby ITS1F and ITS4 pyrosequencing-specific fusion primers that contained GS FLX Titanium A and B adaptor primer sequences, respectively, were added to the amplicons (Figure 1; Table 1). This included the template specific primer sequence added to the 3'-region of the fusion primer to allow for the pyrosequencing emulsion PCR to occur. In addition, each tissue type from the two trees had a specific Multiplex Identifier (MID) sequence added to the ITS1F fusion primers to allow for multiplexing and differentiation according to tissue type (Amplicon Fusion Primer Design Guidelines for GS FLX Titanium Series Lib-A Chemistry, Roche, 2009). PCR conditions were identical to those of the first PCR amplification, except that 2 µl of the purified amplicons of the first PCR were used as template DNA. The PCR products were visualised, gel purified and the concentration of each sample was determined.

The cleaned PCR products were pooled according to tissue type for each tree, resulting in six samples that were submitted for pyrosequencing by Inqaba Biotech for Biotechnology (Pretoria, South Africa). Two pyrosequencing runs were conducted in order to determine if the runs were repeatable. The quality of the sequences obtained from the two pyrosequencing runs were also checked by the diagnostics division of Roche Products (Pty) Ltd, Randburg, South Africa, using Roche Applied Biosystems software (Anonymous 2010).

The pyrosequencing results were manually analysed using a bioinformatics pipeline (Figure 2) developed by Pillay (2012). The sequence files were subjected to an automated filtering step that removed poor quality sequences that were less than 100 base pairs in length as well as chimeras. Sequences less than 100 base pairs reduces the taxonomic resolution for identification purposes (Tedersoo *et al.* 2010; Pompanon *et al.* 2012), whilst

chimeric sequences may lead to the inclusion of non-existent species, thus overestimating the diversity present (Gonzalez *et al.* 2005; Haas *et al.* 2011).

Clustering of similar sequences at a 98 % confidence interval using the CD-HIT package followed (Pillay 2012). Singleton clusters were also removed, because these sequences may result from PCR artefacts or sequencing errors (Tedersoo *et al.* 2010; Kumar *et al.* 2011; Blaalid *et al.* 2012). The longest sequence representing each cluster was used so that Molecular Operational Taxonomic Units (MOTU's) could be assigned to the clusters. Clusters which BLASTed to the same hits were analysed together and neighbour-joining trees were constructed in MEGA version 4.0 (Tamura *et al.* 2007) with representative sequences from a local database and from the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/) GenBank DNA sequence database to identify the MOTU representing the sequences belonging to that cluster.

2.2.2. Statistical analyses

Similarity plots and analyses were carried out using the Vegan package in R 2.14 (Oksanen *et al.* 2012; R Development Core Team 2012). Rarefaction curves based on MOTU richness by sample were used to estimate the overall MOTU diversity and to assess the proportion of the total community represented by the data. The expected number of MOTU's by sampling intensity, with confidence intervals of 95 %, was calculated using EstimateS (Colwell 2000) using 5 000 iterations and rarefying to the minimum number of MOTU's across samples (richness = 8).

2.3. Isolate-based barcoding

2.3.1. DNA extraction and sequencing

The mycelia from the surface of all the pure cultures were scraped and freeze dried for 24 hours. DNA was extracted using a modified CTAB method (Möller *et al.* 1992). After

precipitating the DNA, the supernatant was discarded and the DNA pellet was washed twice with 100 µl of 70 % ethanol and centrifuged at 10 000 rpm for 5 minutes for each wash step. The ethanol was removed and the pellet was left to air dry after the second wash until all ethanol vapours had evaporated. The DNA was re-suspended in 50 µl nuclease-free water and allowed to incubate overnight at 4 ºC (Möller *et al.* 1992). The DNA concentration of each sample was determined using a NanoDrop™ 1000 Spectrophotometer version 3.7 (Thermo Fisher Scientific Inc. NYSE: TMO) and the working stocks were diluted to 40 ng/µl with nuclease-free water.

The full-length ITS of the ribosomal operon region was amplified for each sample. PCR amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the V9G (5'-TTA CGT CCC TGC CCT TTG TA-3') (de Hoog and Gerrits van den Ende 1998) and ITS4 primer pair. The volume for each reaction was 25 µl with 40 ng of template DNA, 10 pmoles of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 1x NH_4 Reaction Buffer (Roche Products Pty Ltd, South Africa), 1.5 mM $MgCl_2$ (Roche Products Pty Ltd, South Africa), 75 µM dNTPs (Roche Products Pty Ltd, South Africa) and 5 units of Fast Start Taq (Roche Products Pty Ltd, South Africa). Nuclease-free water was added to obtain a final volume of 25 µl. The PCR cycling parameters were identical to those in the first round of PCR amplification for the pyrosequencing approach, except that the annealing temperature was at 55 ºC. To verify that the PCR reaction was successful, the PCR products were visualised under UV light using 2 % agarose gel electrophoresis.

The PCR products were cleaned with Sephadex G-50 columns (Steinheim, Germany) to enable direct DNA sequencing. The cleaned PCR product was used as a template for each sequencing reaction performed with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Forster City, CA). Forward and

reverse sequencing were performed with the same primers used for the PCR amplification. The sequencing reactions were subsequently run on an ABI PRISM™ 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and the resultant sequences were manually analysed with CLC Main Workbench version 5.6 (CLC Bio A/S, Aarhus, Denmark) to assemble contigs with both the forward and reverse sequences.

Identities for the resultant contigs were obtained by screening the results in a series of stages (Figure 3) to assess the most accurate identities for the various isolates based on sequences in the NCBI GenBank DNA sequence database (Pillay 2012). In the first stage, sequences were subjected to a BLASTn analysis against the GenBank DNA sequence database, and results were used as an initial grouping step where the sequences were clustered according to their generic affiliations. The sequences were added to a local database using CLC Main Workbench version 5.6. Subsequently, alignments were performed for each cluster using MAFFT version 5.667 (Katoh *et al.* 2005) and base pair differences between isolates were checked manually in MEGA version 4.0. The second stage was a preliminary validation step to determine the number of phylogenetic groups present for each cluster based on manual comparisons aided with neighbour-joining trees with bootstrap analysis, with 1 000 replicates, constructed from the ITS sequences in MEGA version 4.0. Each phylogenetic group was again subjected to BLAST analysis to verify their identities and an attempt was made to identify the unidentified isolates possibly to species level. Where no assignment was possible, the sequences were flagged for further validation against established trees for the particular fungal groups, or to possibly assign the sequences to a family or order based on GenBank DNA sequence database accessions related to the sequences. Phylogenetic units where no species name could be assigned were annotated as distinct or indistinct at a percentage similarity (98 %) cut-off, similar to that used in the pyrosequencing clustering to ensure optimal identification. The final stage of the screening process involved checking the decisions made in the first two

stages. Only sequence accessions from reputed sources were used. The final step will be to submit the sequences obtained as official barcodes to the Barcode of Life Data System (BOLD; www.barcodinglife.org) of the Consortium for the Barcode of Life (CBOL; http://www.barcoding.si.edu/) or other recognised barcoding depositories. This work is in progress as these databases are still being prepared for large numbers of fungal accessions.

3. Results

3.1. Environmental barcoding

The two pyrosequencing runs produced 9 882 and 13 139 sequences, respectively (Table 2). From the quality data, the first run produced a total key pass of 83.99 % with few mixed reads or dot reads, which is an indication of good sequence data (Anonymous 2010). The second run was also of good quality with a key pass of 95.03 %, however, there were a large number of short reads that resulted in the increased number of sequences in this run. When the two runs were compared with each other, they were similar based on alignments. No clusters contained sequences from only one run. This indicates that the runs were repeatable and because of the similarity between the two runs, the sequences were pooled and analysed as a single run.

In total, 454 GS FLX Titanium pyrosequencing produced 23 021 mini-barcodes from the six individually pooled samples (Table 2). With regards to the pyrosequencing data and the corresponding figures and tables that follow, tree 2 will be referred to at tree 1 and tree 3 as tree 2. For the first tree 4 229, 7 386 and 858 sequences were generated for the leaves, twigs and trunk increments, respectively. The leaves of the second tree yielded 3 668 sequences, while 4 728 and 1 152 sequences were generated from the twigs and trunk increments, respectively. The average length of the sequences was 500 base pairs and ranged from 100 – 570 base pairs in length.

After the poor quality sequences and chimeras had been removed, the remaining 6 674 sequences were clustered and grouped into 204 groups based on 98 % similarity, after validation with the isolate-based sequences and reference sequences obtained from the NCBI GenBank DNA sequence database. The clusters which BLASTed to the same hit were analysed together. In total, 92 Molecular Operational Taxonomic Units (MOTU's) were considered valid and represented 15 orders and 20 families. It was not possible to assign names to the MOTU's for 15 % of the groups due to ambiguous assignments or no hit in the databases, and these were assigned numbers (unclassified MOTU 1 – 14). Identification to species level was not done because the complete ITS1 and ITS2 regions were not obtained and results could be misleading (Table 3).

Members of the Ascomycota were dominant and the most represented classes were the Dothidiomycetes and Sordariomycetes. The Basidiomycota was represented by the Agaricomycetes and Tremellomycetes. Species of Mycosphaerellaceae were the most dominant group detected with pyrosequencing (14 %) and represented 13 different MOTU's (Table 3; Figure 4). They were found to be associated with all three tissue types from both trees, with the largest proportion of these occurring in the leaves. MOTU's of *Microdochium* (MOTU 1 – 8) and *Cladosporium* (MOTU 1 – 6), respectively, represented 8.6 % and 6.5 % of the MOTU's. MOTU's of *Degelia*, Tricholomataceae, Pleosporales and *Epicoccum* (all with 5 MOTU's) each presented 5.4 % of the MOTU's, whilst Dothioraceae and *Devriesia* (both with 4 MOTU's) each represented 4.3 % of the MOTU's. MOTU's of *Horteae*, *Periconia* and *Lophiostoma* individually represented 3.2 %, while *Penicillium* and *Usnea* represented 2.2 % of the MOTU's. MOTU's of *Alternaria*, *Pestalotiopsis*, Diaporthales, *Cryptococcus*, *Mycoleptodiscus*, *Neurospora*, *Bionectria*, *Fellowmyces* and *Bullera* each represented one cluster (1.2 %).

The most diverse tissue type in the current study appeared to be the twigs from tree 2 and the leaves of tree 1, each with 46 % and 44 % of the represented MOTU's (Figure 5). The leaves of tree 2, trunk of tree 1 and the trunk of tree 2 had 36.6 %, 12.9 % and 16.1 % of the represented MOTU's, respectively. The twigs from tree 1 had the smallest fungal diversity with 8.6 % of the MOTU's. Of the 92 MOTU's, 7 MOTU's (7.6 %) were found in association with four of the tissue types, 7 MOTU's (7.6 %) were found to be associated with three of the tissue types only, 26 MOTU's (28.3%) were found associated with two tissue types only and 52 MOTU's (56.5 %) were in only one tissue type (Table 3; Figure 6). Of the MOTU's associated with only one tissue type, 42.3 % were associated with the leaves only, whilst 9.6 % and 48.1 % of the MOTU's were only found in the trunk and twigs, respectively. This relatively low level of overlap may be an indication that the MOTU's show some degree of tissue specificity.

Nonmetric Multidimensional Scaling (NMDS) ordination spider plots were used to capture the overlap in community structure between and among trees (Figure 7A) and the overlap between and among tissues (Figure 7B). Tissue data could not be analysed statistically as only two trees were pyrosequenced. While not statistically significant using an Adonis analysis of similarity ($F_{1.4} = 0.89$; $p = 0.7802$; $R^2 = 0.18$), the visual lack of overlap between the trees in ordination space hints at the possibility that the endophyte community may be more similar within trees than across trees, as might be expected (Figure 7A). It also appears that there may be moderate tissue specificity as twigs grouped away from leaves and trunk increments in multivariate space, though more sampling is required to elucidate such trends (Figure 7B).

The rarefaction curves generated appear to be approaching saturation and it appears that well over half of the endophytic community has been sampled (Figure 8). However, since only two trees were sampled from a single site, the scope of the diversity estimates in this

study is quite limited. The degree to which endophyte communities vary across the individual trees, genotypes and geographical locations remains an interesting and important question for which substantial additional sampling is required.

3.2. Isolate-based barcoding

Based on BLAST results, the isolates represented 13 orders, 22 families and 42 different species (Figure 9). Not all isolates could be identified to species level because the ITS ribosomal DNA region is known to have insufficient resolution between the different species in certain groups, such as *Penicillium*, *Pestalotiopsis*, *Cladosporium* and Xylariaceae. *Pestalotiopsis* sp. 2 was the most dominant fungal endophyte isolated and represented 33.2 % of all the isolates. Other dominant genera included *Bionectria ochroleuca* (7.2 %), *Colletotrichum* aff. *gloeosporioides* (5.6 %), *Neofusicoccum mediterraneum* (5.6 %) and *Pestalotiopsis* sp. 1 (4.4 %). Single isolates made up 4.8 % of the total number of individuals. Approximately 12 % of the isolates were represented by five unknown species (unknown spp. 1 – 5) that could not be adequately identified. The reason that these are unknown is most likely because they could not be assigned to any known genus, family or order satisfactorily, or they grouped to GenBank DNA sequence database accessions excessively populated by sequences generated by environmental approaches. These sequences could represent largely unclassified fungal groups or unculturable fungi.

3.3. Comparison of sequences obtained from environmental and isolate-based barcoding

The results obtained for the environmental and isolate-based barcoding data was compared in order to determine the level of overlap between them. As the ITS mini-barcodes generated by 454 FS GLX Titanium pyrosequencing were only partial

sequences, comparing them to the full-length sequences generated from the isolate-based barcoding was only possible to family and genus levels. Families that were shared included the Amphispheriaceae (*Pestalotiopsis* spp. 1 and 2; *Pestalotiopsis* MOTU), Bionectriaceae (*Bionectria ochroleuca*; *Bionectria* MOTU), Cladosporiaceae (*Cladosporium delicatulum*; *Cladosporium* MOTU 1 – 6), Dothioraceae (*Sydowia eucalypti*; Unknown genus MOTU 1 – 4 and *Hortaea* MOTU 1 – 3), Magnaporthaceae (Magnaporthales sp; *Mycoleptodiscus* MOTU), Mycosphaerellaceae (*Mycosphaerella marksii*, *M. vietnamensis*, *Pseudocercospora crystallina* and a *Septoria* sp.; *Mycosphaerella* MOTU 1 – 13), Pleosporaceae (*Alternaria alternate*, *Leptosphaerulina chartarum*, *Periconia macrospinosa* and a Pleosporales sp; *Alternaria* MOTU and *Pleospora* MOTU 1 – 5), Sorderiaceae (*Neurospora crassa*; *Neurospora* MOTU) and Trichocomaceae (*Penicillium* spp. 1 – 3; *Penicillium* MOTU 1 and 2). The genera that could be conclusively identified from environmental barcoding and found with both techniques were *Alternaria*, *Bionectria*, *Penicillium* and *Pestalotiopsis*. It was not possible to compare Mycosphaerellaceae, Cladosporiaceae, or many other families further than family level as more gene regions are required to delineate the species in these groups.

4. Discussion

Both the isolate-based barcoding and environmental barcoding approaches were used to characterise the endophytic diversity of *Syzygium cordatum*. Two hundred and fifty isolates were sequenced and these represented 13 orders, 22 families and 42 species. This is compared to the 15 orders, 20 families and 92 Molecular Operational Taxonomic Units (MOTU's) from 6 674 filtered reads found after 454 GS FLX Titanium pyrosequencing for the environmental barcoding portion of this study.

The dominant fungi isolated in this study were two species of *Pestalotiopsis*. Pestalotoid fungi have been isolated as endophytes in various studies and have been reported as

pathogens as well as saprophytes (Cannon and Simmons 2002; Photita *et al.* 2004; Hyde *et al.* 2007; Wei *et al.* 2007). Due to the dominance of this group of fungi in culture, one would have expected this dominance to be reflected in the environmental barcoding analysis. However, only one MOTU of *Pestalotiopsis* was detected with pyrosequencing. Similar findings were also presented by Pillay (2012) and this indicates that culture-based methods may be deceptive and over-estimate the dominance or absence of some fungi (Evans and Seviour 2011). This could be due to the presence of slow growing fungal species, such as *Mycosphaerella*, which would be out competed by species that may be rare, but that grow rapidly in culture (Cannon and Simmons 2002).

The dominant group of endophytes detected with pyrosequencing were species of Mycosphaerellaceae, which are well-known and important leaf and shoot pathogens of *Eucalyptus* trees and other native plant species in South Africa (Hunter *et al.* 2004a; Hunter *et al.* 2004b; Crous *et al.* 2006; Bensch *et al.* 2010; Hunter *et al.* 2011). Isolates of *Pseudocercospora*, *Septoria* and *Mycosphaerella* were detected in this study based on the full-length sequences of the Internally Transcribed Spacer of the nuclear ribosomal DNA operon. This group is known to include endophytes, pathogens and saprobes (Hunter *et al.* 2004b; Hunter *et al.* 2006; Crous *et al.* 2009; Hunter *et al.* 2011), but the common occurrence of species of the Mycosphaerellaceae as one of the dominant and diverse groups of endophytes from *S. cordatum* was surprising. The same pattern was observed in a parallel study conducted by Pillay (2012). It would be interesting to determine whether similar patterns of dominance occur across other geographical locations, and possibly over a range of other hosts. This could be important for disease control, because some of these fungi are well-known pathogens on *Syzygium*, and also *Eucalyptus* trees, in various parts of the world (Crous and Wingfield 1991; Carnegie and Keane 1994; Crous and Wingfield 1996; Carnegie *et al.* 1997; Carnegie and Keane 1998; Crous 1999; Maxwell *et al.* 2003; Hunter *et al.* 2004a; Burgess *et al.* 2007; Pérez *et al.* 2009).

It was interesting that only species of Mycosphaerellaceae and Cladosporiaceae were isolated from *S. cordatum* in this study, and not members of the closely related Teratosphaeriacaeae, that include numerous pathogens of closely related *Eucalyptus* spp. (Crous *et al.* 2007a; Andjic *et al.* 2010; Andjic *et al.* 2011). Only *Mycosphaerella marksii*, *M. vietnamensis*, *Cladosporium delicatulum*, *Pseudocercospora crystallina* and a species of *Septoria* were isolated and 13 MOTU's of *Mycosphaerella* and 6 *Cladosporium* MOTU's were detected using pyrosequencing. Only 4 *Devriesia* MOTU's representing the Teratosphaeriaceae were found with pyrosequencing. However, sampling was limited and not aimed to completely sample the endophyte diversity across a larger number of trees, and numerous species were most likely missed. Future surveys would thus be necessary to confirm this trend.

The Botryosphaeriaceae from *S. cordatum* in South Africa have been extensively studied (Pavlic *et al.* 2004; Pavlic *et al.* 2007b; Pavlic *et al.* 2007a; D. Pavlic *et al.* 2009). These include both those species associated with disease symptoms and those isolated as endophytes from healthy tissue. Eight species are known from *S. cordatum* with *Lasidioplodia theobromae*, *Neofusicoccum kwambonambiensis*, *N. mangiferae*, *Neofusicoccum parvum*, *N. ribis* and *N. umdonicola* known from the same region where the samples were obtained in the present study (Pavlic *et al.* 2007b; D. Pavlic *et al.* 2009). Very few Botryosphaeriaceae were isolated in this study, with only a species of *Lasidioplodia* and *N. mediterraneum* identified from BLAST results. This group was also not detected with pyrosequencing, but more sampling should be done to confirm this unexpected result.

It was not possible to identify species of Mycosphaerellaceae, Cladosporiaceae and Botryosphaeriaceae satisfactorily based on the sequence data obtained. For both these groups, multi-gene analyses are necessary to identify taxa to species level. For the

Mycosphaerellaceae and closely related Teratosphaeriaceae, sequences from the Large Subunit (LSU) gene region are usually coupled with ITS sequences, and large DNA sequence datasets exist where species can be compared to all currently valid and described species with phylogenetic analyses (Hunter *et al.* 2006; Crous *et al.* 2007a; Arzanlou *et al.* 2008; Crous 2009). Translation Elongation Factor-1α (TEF-1α) sequences are used if greater resolution is required (Hunter *et al.* 2006; Crous *et al.* 2007a; Crous 2009). Actin (ACT) and TEF-1α need to supplement the ITS sequence for species identification for the Cladosporiaceae. For the Botryosphaeriaceae, ITS sequences are usually augmented with housekeeping genes, such as β-tubulin-2 (βt 2a/b), EF-1α, and the RNA polymerase II subunit (RPB2) gene sequences, because ITS is known to not distinguish between some species of the Botryosphaeriaceae (Smith *et al.* 2001; Slippers *et al.* 2004; D. Pavlic *et al.* 2009; Draginja Pavlic *et al.* 2009). However, this was not the scope of this study and more complete phylogenetic analyses will be done to accurately identify these important pathogen groups from *S. cordatum* [Pillay (2012) for the Botryosphaeriaceae, and **Chapter 3** of this thesis for the Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae].

A number of plant pathogens were isolated as endophytes and detected with pyrosequencing. Besides the Mycosphaerellaceae and Botryosphaeriaceae, *Sydowia eucalypti* was isolated as an endophyte in this study and has previously been reported from leaf spots on *Eucalyptus* (Park and Keane 1984; Crous 1998; Crous *et al.* 2007b; Sánchez Márquez *et al.* 2010). *Colletotrichum* aff. *gloeosporioides* and *Alternaria alternata* were detected in the isolations of this study and are known to be species complexes including pathogens of numerous agricultural crops (Cameron *et al.* 1997; Novas and Carmarán 2008; Andrew *et al.* 2009). However, these isolates of *Alternaria* and *Colletotrichum* need to be subjected to the latest multi-gene analyses to be

appropriately identified and to determine if they are identical to those species that are pathogens.

Eight *Microdochium* (teleomorph *Monographella*) MOTU's were detected with pyrosequencing and this genus contains pathogens of grasses (Kwasna and Bateman 2007; Kammoun *et al.* 2009). A MOTU of *Diaporthe*, a genus that includes numerous canker pathogens of tree species (Smit *et al.* 1996; Santos and Phillips 2009) was also detected with the pyrosequencing. No species identities could be derived for the *Microdochium* and *Diaporthe* species based on the partial ITS sequences obtained in the pyrosequencing.

A very high diversity of endophytes was found to be associated with *Eucalyptus grandis* x *E. camaldulensis* clones originating from the same area where the *S. cordatum* was sampled in this study (Pillay 2012). In the study of Pillay (2012), 85 species were identified from the isolate-based barcoding approach, representing 19 orders and 40 families and from the environmental barcoding approach, 1 280 MOTU's were identified which represented 14 orders and 28 families. This is contrasted with the 42 species residing in 13 orders and 22 families that were isolated and identified from *S. cordatum*, and the 92 MOTU's representing 15 orders and 20 families using the environmental barcoding approach. The two tree species were sampled at the same time, in the same location using identical sampling and processing protocols (M. Gryzenhout, personal communication). It has been thought that native trees could harbour more endophytic diversity than non-native trees (Fisher *et al.* 1993; Fisher *et al.* 1994; Mitchell and Power 2003; Levine *et al.* 2004; Hoffman and Arnold 2008). However, in this case, the results from this study and that of Pillay (2012) suggest that the opposite might be true.

There could be three reasons for finding fewer species of endophytes infecting *Syzygium cordatum* compared to those from *E. grandis* (Pillay 2012). The fact that the DNA from

tree 1 in this study was degraded and could not be amplified successfully for pyrosequencing the nested PCR, may indicate that the DNA from trees 2 (tree 1 in the pyrosequencing analyses) and 3 (tree 2 in the pyrosequencing analyses) were also in the process of degradation. Hence not all species present were amplified. The samples and DNA were, however, treated in the same way as those of the *Eucalyptus* clone. The second possibility is that the pyrosequencing runs were unsuccessful. However, the runs were repeated, showing similar results with good quality score data. The third possibility is that the lower endophyte diversity could be an accurate reflection of the diversity found in *S. cordatum* compared to that associated with the *E. grandis* clone in South Africa. The fact that occasionally non-native plants harbour more diverse communities of fungi than introduced plants has been found in other studies (Fisher *et al.* 1993; Fisher *et al.* 1994; Hoffman and Arnold 2008; Shipunov *et al.* 2008). The increased number of endophytes in non-native trees could thus be due to the occurrence of both native and introduced endophytes present. The increased number of endophytes may also be due to the presence of generalist endophytes that lack host specificity and are common in plants outside their native ranges (Fisher *et al.* 1994; Hoffman and Arnold 2008; Shipunov *et al.* 2008). Further studies on *S. cordatum* and *Eucalyptus* spp. would be necessary to confirm these findings.

This study provided an important foundation towards understanding the fungal diversity associated with *S. cordatum*. Furthermore, the information gathered contributes to the body of knowledge available on fungi of South Africa. More extensive studies can now be based on this information in the future to thoroughly investigate the fungal diversity associated with these native South African trees. It is clear, even from this study, that only the tip of the iceberg has been explored in terms of fungal diversity. The local database developed in this study based on isolate-based barcoding greatly aided in validating the results obtained from the pyrosequencing analysis. It is important, however, to note that

the endophytic distributions are heavily influenced by the depth of sequencing and sampling and will change with more in depth analyses.

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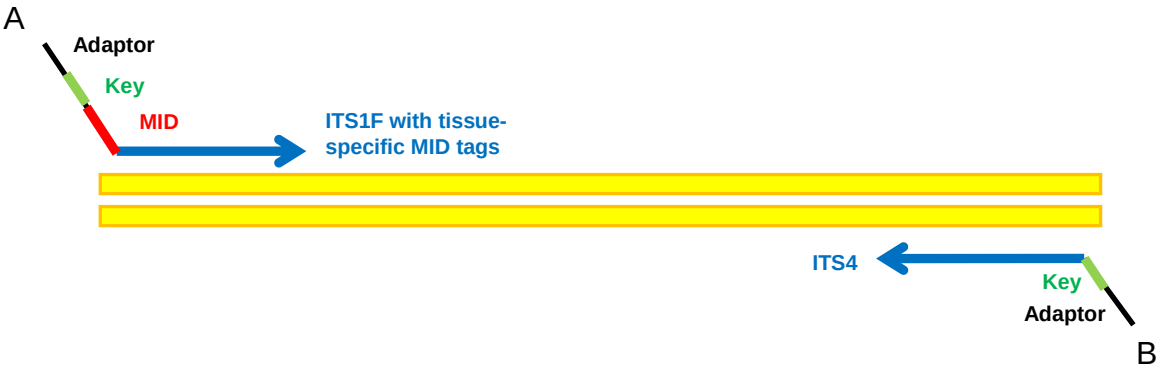

Figure 1: Design of amplicon fusion primers for 454 GS FLX Titanium pyrosequencing (adapted from the 454 Sequencing Technical Bulletin)

Table 1: Tissue-specific amplicon fusion primers for pyrosequencing

Tree	Tissue	Primer Sequence
1	Leaves	5'-CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGcttggtcatttagaggaagtaa-3'
	Twigs	5'-CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGcttggtcatttagaggaagtaa-3'
	Trunk Increments	5'-CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTActtggtcatttagaggaagtaa-3'
2	Leaves	5'-CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGcttggtcatttagaggaagtaa-3'
	Twigs	5'-CGTATCGCCTCCCTCGCGCCATCAGTAGTATCAGCcttggtcatttagaggaagtaa-3'
	Trunk Increments	5'-CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGcttggtcatttagaggaagtaa-3'
ITS4 with B adaptor		5'-CTATGCGCCTTGCCAGCCCGCTCAGtcctccgcttattgatatgc-3'

Colour indicators: *Adaptor (black); Key (**green**); MID (**red**); ITS1F or ITS4 primer sequence (**blue**).*

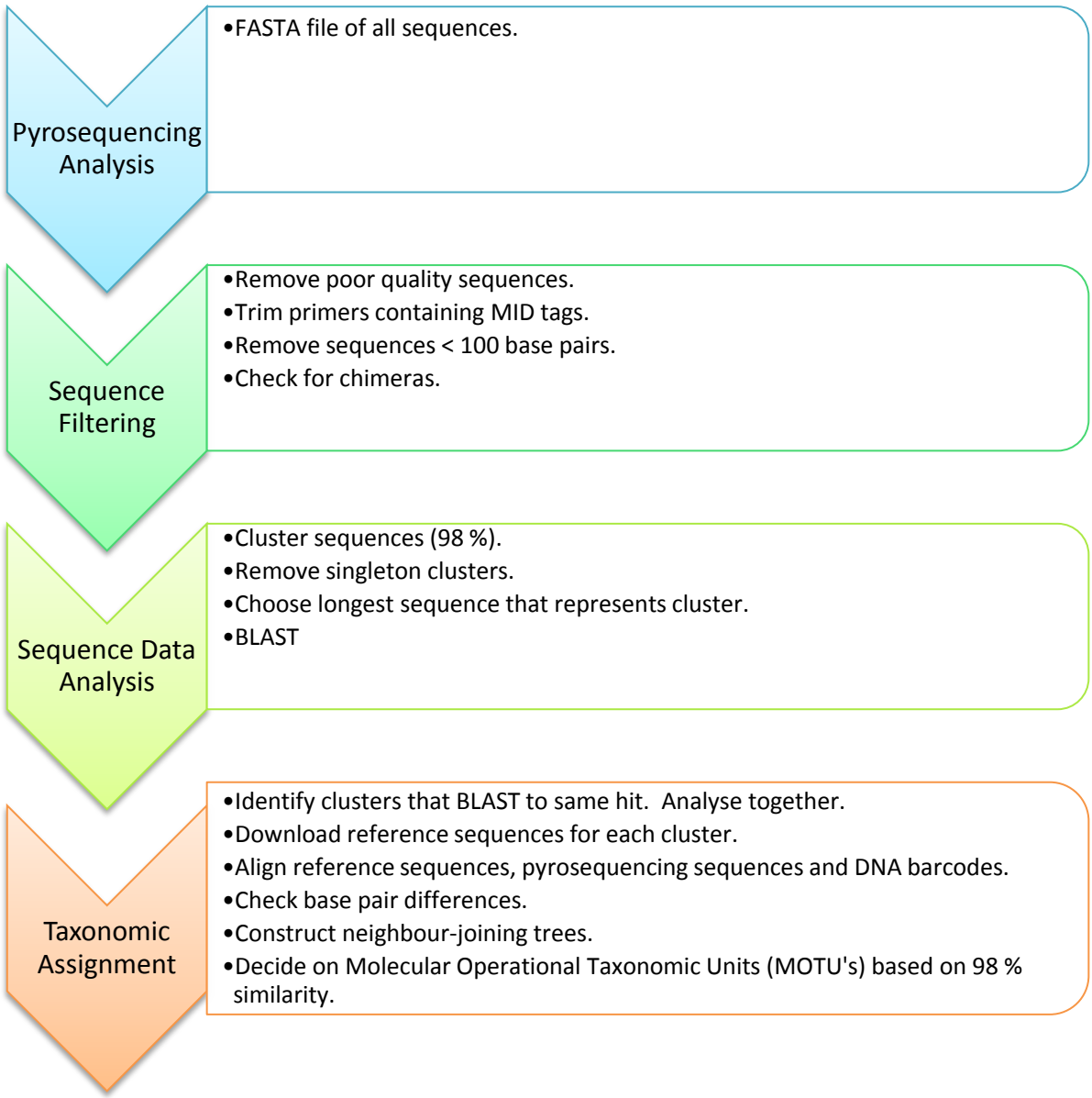

Figure 2: Pipeline developed by Pillay (2012) for the analysis of 454 GS FLX Titanium pyrosequencing data. The first three steps are automated and the final taxonomic assignment step is manual.

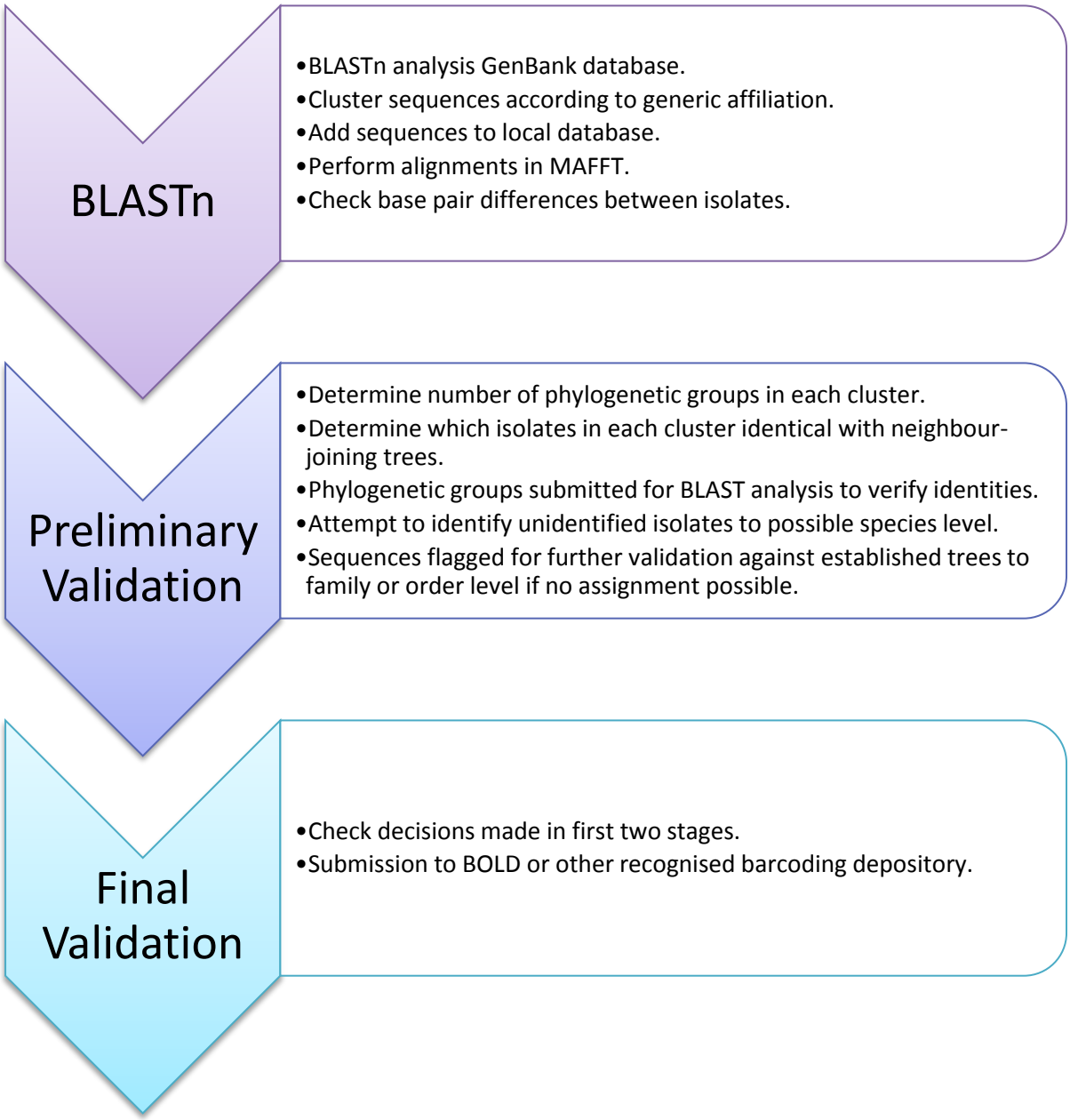

Figure 3: Taxonomic approach developed by Pillay (2012) to assess the most accurate identities for the various isolates obtained for barcoding.

Table 2: Total number of sequences generated and number of sequences analysed per tissue and classification of Molecular Operational Taxonomic Units (MOTU's) generated by environmental barcoding.

Tissue	No. of sequences	No. of sequences analysed	Phyla	Classes	Orders	Families	Genera	MOTU's
B1	858	212	1	3	7	8	7	12
L1	4 229	1 899	2	4	9	10	8	41
T1	7 386	1 091	1	2	2	2	2	8
B2	1 152	284	2	3	6	8	7	15
L2	3 668	785	2	3	9	14	13	34
T2	4 728	2 403	2	5	14	19	17	43
Summary	**21 021**	**6 674**	**2**	**6**	**15**	**20**	**24**	**92**

Key: *B1/B2 = Trunk increments with bark attached of tree 1 / 2; L1/L2 = Leaves of tree 1 / 2; T1/T2 = Twigs of tree 1 / 2*

Table 3: Molecular Operational Taxonomic Units (MOTU's) found in each tissue type with pyrosequencing.

Phylum	Class	Order	Family	Genus	MOTU	B1	L1	T1	B2	L2	T2
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	MOTU 1		●		●	●	●
				Cladosporium	MOTU 2	●					
				Cladosporium	MOTU 3						●
				Cladosporium	MOTU 4		●			●	●
				Cladosporium	MOTU 5		●			●	
				Cladosporium	MOTU 6		●			●	
			Mycosphaerellaceae	*Mycosphaerella*	MOTU 1	●	●			●	●
				Mycosphaerella	MOTU 2		●				
				Mycosphaerella	MOTU 3		●	●	●		
				Mycosphaerella	MOTU 4		●	●			
				Mycosphaerella	MOTU 5						●
				Mycosphaerella	MOTU 6		●				
				Mycosphaerella	MOTU 7		●				
				Mycosphaerella	MOTU 8		●				
				Mycosphaerella	MOTU 9		●			●	
				Mycosphaerella	MOTU 10		●			●	
				Mycosphaerella	MOTU 11		●				
				Mycosphaerella	MOTU 12				●	●	●
				Mycosphaerella	MOTU 13						●
			Teratosphaeriaceae	*Devriesia*	MOTU 1				●		●
				Devriesia	MOTU 2		●			●	
				Devriesia	MOTU 3		●				●
				Devriesia	MOTU 4					●	●

	Incertae sedis	*Incertae sedis*	*Epicoccum*	MOTU 1	●	●				●
			Epicoccum	MOTU 2	●				●	
			Epicoccum	MOTU 3		●				
			Epicoccum	MOTU 4					●	
			Epicoccum	MOTU 5		●				
	Dothideales	Dothioraceae	-	MOTU 1		●				
			-	MOTU 2	●	●		●	●	
			-	MOTU 3		●				
			-	MOTU 4		●				●
			Hortaea	MOTU 1		●			●	
			Hortaea	MOTU 2		●			●	
			Hortaea	MOTU 3		●				●
	Pleosporales	Pleosporaceae	*Pleospora*	MOTU 1	●					
			Pleospora	MOTU 2				●	●	●
			Pleospora	MOTU 3					●	
			Pleospora	MOTU 4		●		●		
			Pleospora	MOTU 5	●				●	
			Alternaria	MOTU		●		●		●
		Lophiostomataceae	*Lophiostoma*	MOTU 1						●
			Lophiostoma	MOTU 2						●
			Lophiostoma	MOTU 3						●
Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	MOTU 1						●
			Penicillium	MOTU 2						●
Lecanoromycetes	Lecanorales	Parmeliaceae	*Usnea*	MOTU 1		●			●	
			Usnea	MOTU 2		●				

		Peltigerales	Pannariaceae	*Degelia*	MOTU 1							●
				Degelia	MOTU 2							●
				Degelia	MOTU 3							●
				Degelia	MOTU 4							●
				Degelia	MOTU 5	●						●
	Sordariomycetes	Diapothales	Diaporthaceae	*Diaporthe*	MOTU							●
		Hypocreales	Bionectriaceae	*Bionectria*	MOTU	●	●			●		
		Magnaporthales	Magnaporthaceae	*Mycoleptodiscus*	MOTU	●						●
		Microascales	Halosphaeriaceae	*Periconia*	MOTU 1							●
				Periconia	MOTU 2							●
				Periconia	MOTU 3							●
		Sordariales	Sordariaceae	*Neurospora*	MOTU					●		
		Xylariales	Amphispheriaceae	*Pestalotiopsis*	MOTU		●		●	●	●	
			Hyponectriaceae	*Microdochium*	MOTU 1			●				●
				Microdochium	MOTU 2		●	●	●			●
				Microdochium	MOTU 3			●				
				Microdochium	MOTU 4			●				●
				Microdochium	MOTU 5			●		●		
				Microdochium	MOTU 6			●				
				Microdochium	MOTU 7		●	●				
				Microdochium	MOTU 8							●
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	-	MOTU 1				●			
				-	MOTU 2		●		●	●		●
				-	MOTU 3							●
				-	MOTU 4							●

						B1	B2	L1	L2	T1	T2
				-	MOTU 5						●
	Tremellomycetes	Tremellales	Cuninulitremaceae	*Fellomyces*	MOTU				●		
			Tremellaceae	*Bullera*	MOTU				●		
				Cryptococcus	MOTU						●
-	-	-	-	Unclassified	MOTU 1			●			●
				Unclassified	MOTU 2			●			
				Unclassified	MOTU 3			●			
				Unclassified	MOTU 4				●		
				Unclassified	MOTU 5	●		●		●	●
				Unclassified	MOTU 6					●	
				Unclassified	MOTU 7						●
				Unclassified	MOTU 8						●
				Unclassified	MOTU 9			●			●
				Unclassified	MOTU 10						●
				Unclassified	MOTU 11			●			
				Unclassified	MOTU 12			●			●
				Unclassified	MOTU 13						●
				Unclassified	MOTU 14						●
				Unclassified	MOTU 15	●					

Key: *B1 / B2 = Trunk increments with bark attached of tree 1 / 2 (red dots); L1 / L2 = Leaves of tree 1 / 2 (green dots); T1 / T2 = Twigs of tree 1 / 2 (blue dots)*

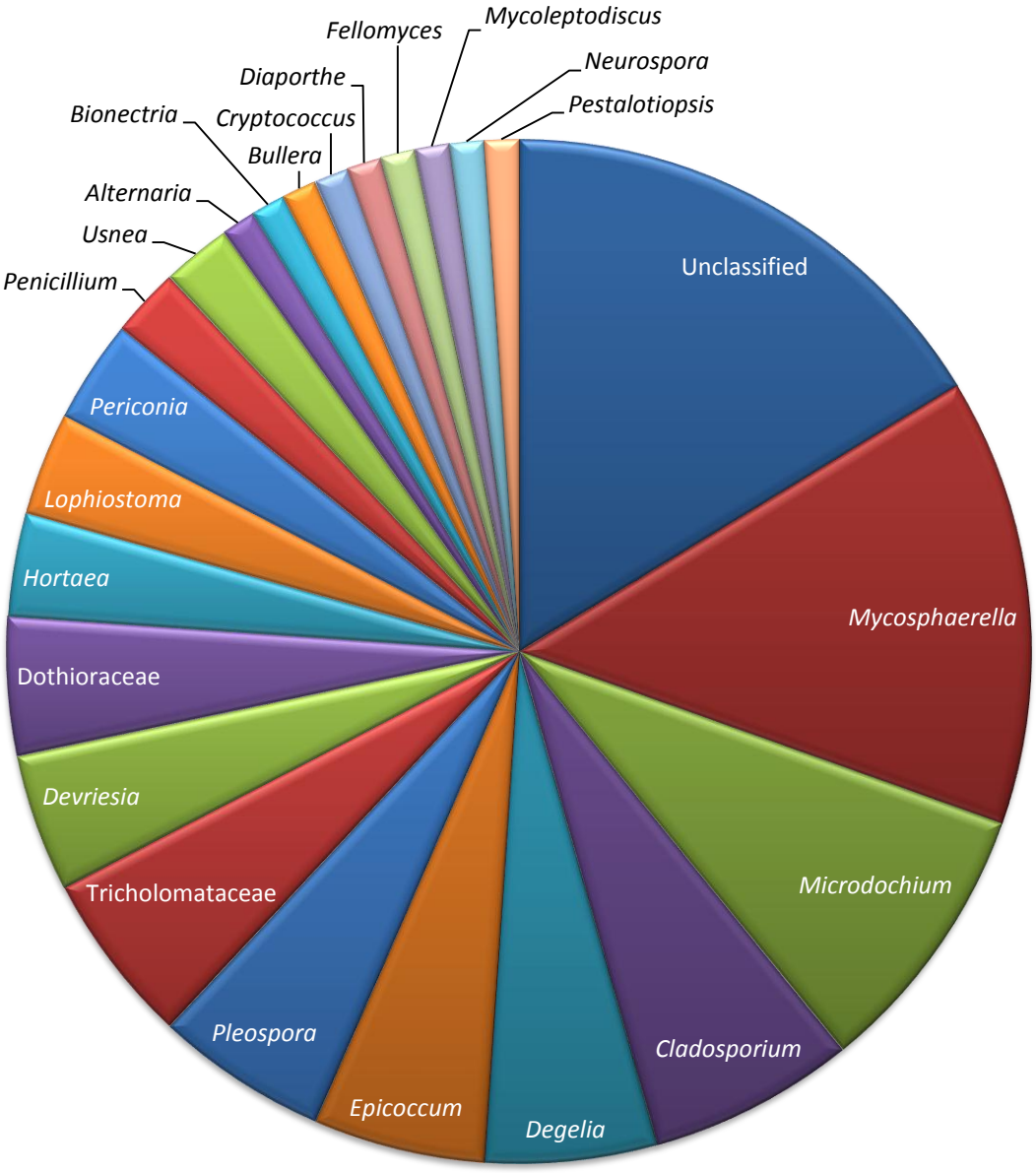

Figure 4: Taxonomic distribution of the 92 Molecular Operational Taxonomic Units (MOTU's) in accordance to the top BLAST hits from the NCBI GenBank DNA sequence and local databases.

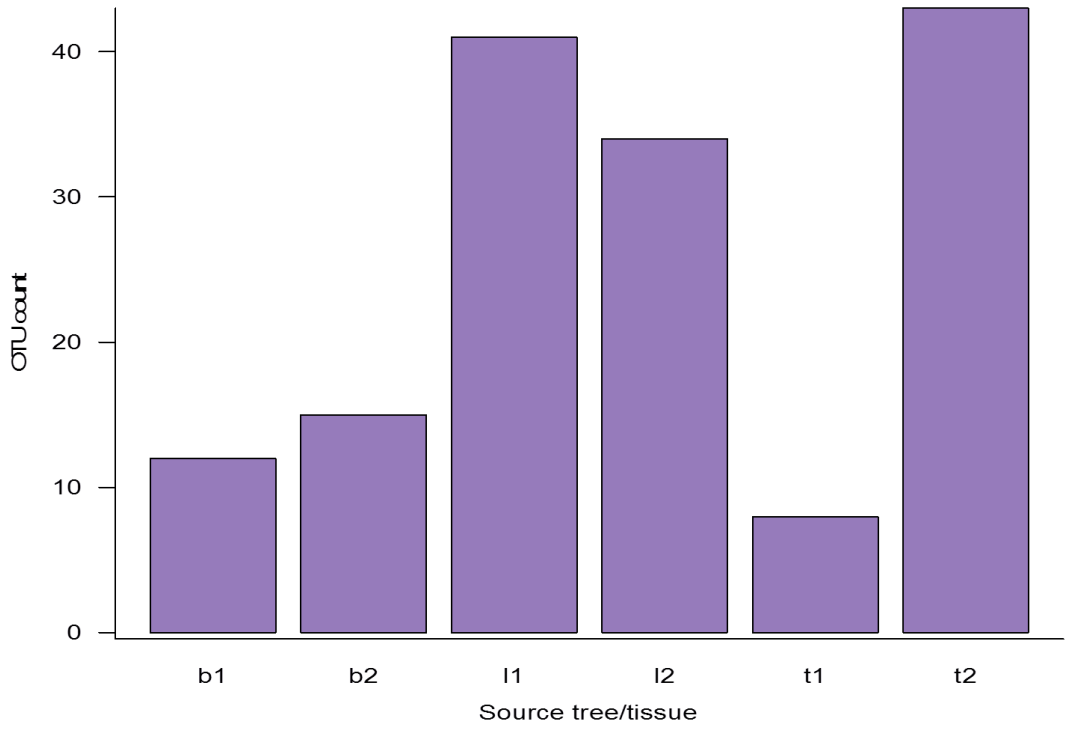

Figure 5: Molecular Operational Taxonomic Unit (MOTU) richness in the different tissues sampled. The twigs of tree 2 and the leaves of tree 1 were the most diverse tissues while the twigs of tree 1 were the least diverse tissue. ***Key:*** *b1 / b2 = Trunk increments with bark attached of tree 1 / 2; l1 / l2 = Leaves of tree 1 / 2; t1 / t2 = Twigs of tree 1 / 2*

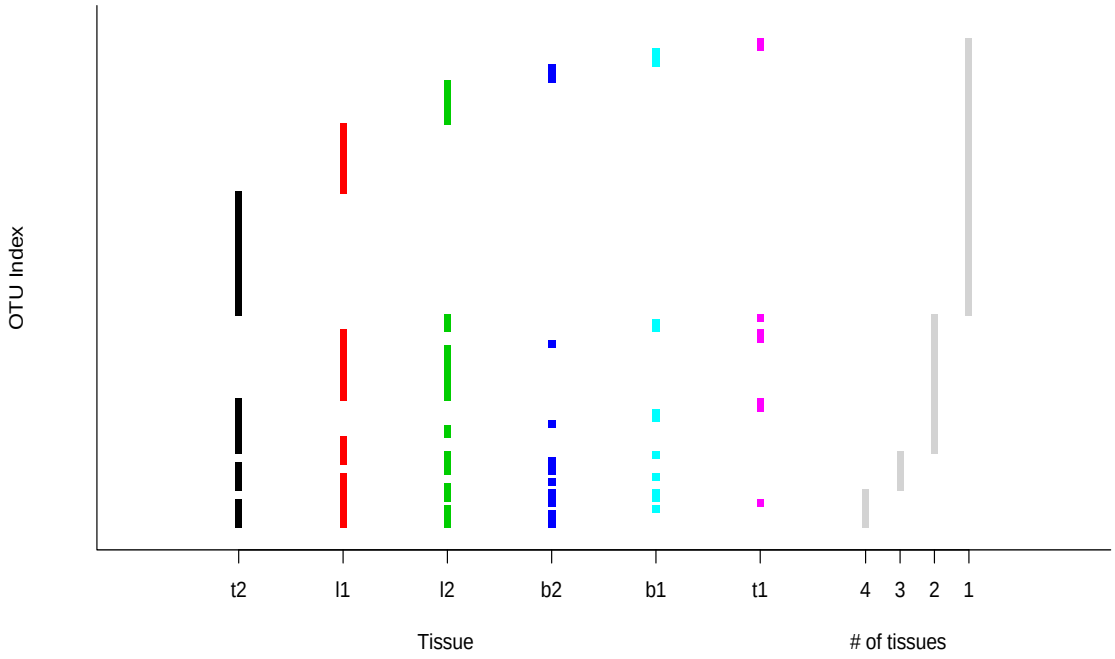

Figure 6: Overlap of Molecular Operational Taxonomic Units (MOTU's) between the different tissues of *Syzygium cordatum*. The various colours represent the MOTU's in each tissue and give a visual indication to the level of MOTU overlap between tissues. Seven MOTU's are found in 4 tissue types, 7 MOTU's are found in 3 tissue types, whilst 26 MOTU's are found in 2 tissue types and 52 MOTU's are only found in 1 tissue type. ***Key:*** *b1 / b2 = Trunk increments with bark attached of tree 1 / 2; l1 / l2 = Leaves of tree 1 / 2; t1 / t2 = Twigs of tree 1 / 2*

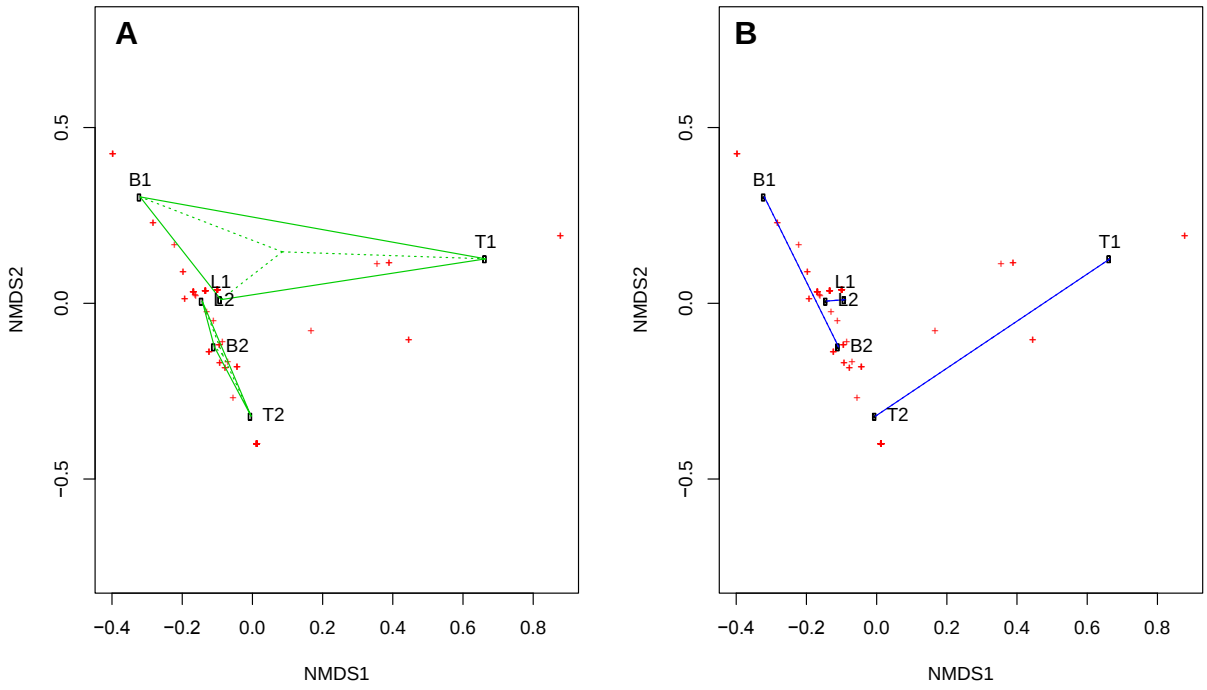

Figure 7: Non-metric multidimensional scaling (NMDS) ordination spider plots showing the level of overlap between and among the two trees (**A**) and and between and among tissues (**B**). The red points on the plots represent the various Molecular Operational Taxonomic Units (MOTU's). The lack of overlap between the trees indicates that the endophytic community within trees than across the trees (**A**) whilst there appears to be moderate tissue specificity (**B**). ***Key:*** *B1 / B2 = Trunk increments with bark attached of tree 1 / 2; L1 / L2 = Leaves of tree 1 / 2; T1 / T2 = Twigs of tree 1 / 2*

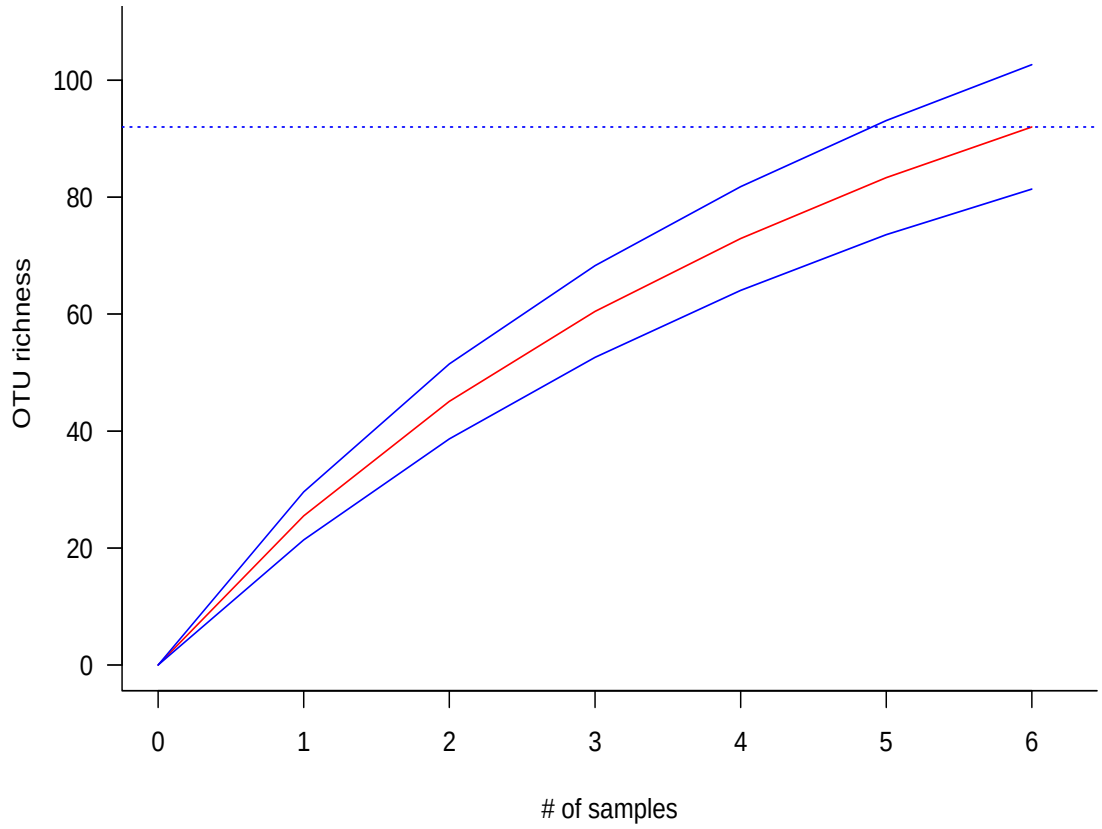

Figure 8: Rarefaction curves generated for the Molecular Operational Taxonomic Units (MOTU's) obtained from the environmental barcoding of the endophytes associated with *Syzygium cordatum*. The curves appear to be approaching saturation and it appears that over half the endophytic community has been sampled. The blue lines represent 95 % confidence intervals and the red line represents the minimum number of MOTU's across the samples.

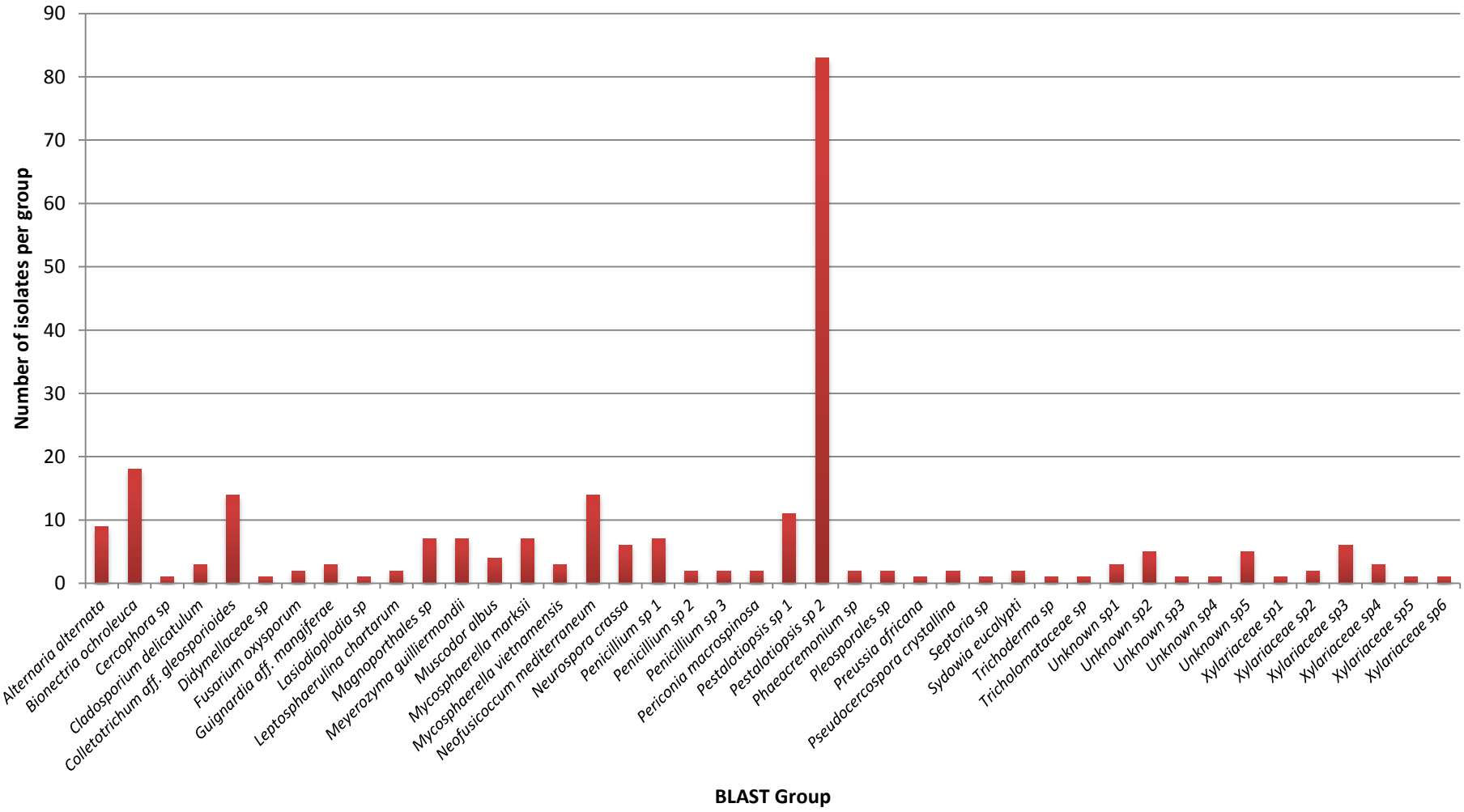

Figure 9: Endophyte diversity identified with isolate-based barcoding.

Unique, dominant and diverse Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae endophytes from Myrtaceae in South Africa

Abstract

Species in Mycosphaerellaceae and Teratosphaeriaceae cause the well-known Mycosphaerella leaf blotch diseases of *Eucalyptus* species in various countries around the world. Many species of *Mycosphaerella* and *Teratosphaeria* are known to occur as asymptomatic endophytes and are known to be latent pathogens or saprobes. During isolate-based barcoding studies of the endophytes associated with a healthy native *Syzygium cordatum* tree and a healthy non-native *Eucalyptus grandis* x *Eucalyptus camaldulensis* clone in South Africa, isolates of the Mycosphaerellaceae, Teratosphaeriaceae and related Cladosporiaceae were among the most commonly isolated groups. Multi-locus sequence analysis using the Internally Transcribed Spacer (ITS) ribosomal DNA, partial Large Subunit (LSU; 28S nrDNA) nuclear ribosomal DNA operon and Translation Elongation Factor-1α (TEF-1α) genes was employed to identify these isolates. Isolates grouped in eight clades representing *Pseudocercospora crystallina* isolated from both trees, *Readeriella considenianae* from the *Eucalyptus* trees and *Cladosporium delicatulum*, *Mycosphaerella marksii* and *M. vietnamensis* from *S. cordatum*. The serious canker pathogen *Teratosphaeria zuluensis* was also abundantly isolated from *Eucalyptus* leaves. Two new species associated with the *Eucalyptus* clone were identified and are described as *Cladosporium impi* prov. nom. and *Mycosphaerella picanini* prov. nom., respectively. Of the species found, *R. considenianae*, *C. delicatulum* and *M. vietnamensis* were isolated in South Africa for the first time, while *M. marksii*, *M. vietnamensis, C. delicatulum* and *Pseudocercospora crystallina* were shown to naturally infect *S. cordatum* for the first time.

1. Introduction

Species in the Teratosphaeriaceae and Mycosphaerellaceae (Ascomycetes) previously resided in the single genus, loosely referred to as *Mycosphaerella sensu lato* (*Capnodiales*; *Dothideomycetes*). This group of fungi includes approximately 3 000 species names representing endophytes, pathogens and saprobes (Corlett 1995; Crous *et al.* 2000; Hunter *et al.* 2004b; Hunter *et al.* 2006; Crous *et al.* 2009d; Hunter *et al.* 2011). Fungi in these genera include important leaf and shoot pathogens of *Eucalyptus*, as well as other trees in the Myrtales, and collectively cause the disease known as Mycosphaerella leaf blotch [MLB] (Crous *et al.* 1991; Hyde *et al.* 2007; Hunter *et al.* 2009; Sánchez Márquez *et al.* 2010). Symptoms of MLB include necrotic leaf lesions that reduce the photosynthetic capacity of the plant and they can lead to defoliation and stem and trunk cankers. This results in multi-stemmed trees not ideal for the paper industry (Hunter *et al.* 2004b; Crous *et al.* 2006b).

Morphological identification is difficult for species of Mycosphaerellaceae and Teratosphaeriaceae, as they grow poorly in culture and the fruiting structures are small and morphologically conserved (Hunter *et al.* 2006). Anamorph genera are morphologically variable compared to the sexual states, and are thus more informative when trying to distinguish between species (Crous 1998; Crous *et al.* 2001a; Hunter *et al.* 2004b). Characteristics used for identification include the presence or absence of aerial mycelium, conidiophores, conidiogenous cells and the conidial morphology.

DNA-based techniques have revolutionised the identification of species within Mycosphaerellaceae and Teratosphaeriaceae. DNA sequence data from the Internally Transcribed Spacer (ITS) ribosomal DNA region has most frequently been used (Crous *et al.* 2001a; Crous *et al.* 2001b; Hunter *et al.* 2004b; Hunter *et al.* 2006). Application of multi-gene analysis using the ITS region, Large Subunit (LSU) of the nuclear ribosomal

DNA operon, Translation Elongation Factor-1α (TEF-1α) and Actin (Act) genes showed that the Mycosphaerellaceae and Teratosphaeriaceae were polyphyletic (Crous *et al.* 2001b; Hunter *et al.* 2006; Crous 2009). Consequently, these families have been divided to include the Cladosporiaceae with mostly *Cladosporium* anamorphs (Schubert *et al.* 2007; Bensch *et al.* 2010), Dissoconiaceae with *Dissoconium* and *Ramichloridium* anamorphs (Crous *et al.* 2009a) and Teratosphaeriaceae and Mycosphaerellaceae that now contain most of the previous anamorph genera of *Mycosphaerella sensu lato* (Crous *et al.* 2007; Crous *et al.* 2009e). The anamorph genera in the Mycosphaerellaceae include *Cercospora*, *Cercosporella*, *Dothistroma*, *Lecanosticta*, *Miuraea*, *Passalora*, *Periconiella*, *Phaeophleospora*, *Phloeospora*, *Polythrincium*, *Pseudocercosporella*, *Ramulispora*, *Ramularia*, *Rasutoria*, *Septoria*, *Sonderhenia*, *Trochophora*, *Verrucisporota* and *Zasmidium* (Crous *et al.* 2009c). Those in the Teratosphaeriaceae include *Batcheloromyces*, *Baudoinia*, *Capnobotryella*, *Catenulostroma*, *Davisoniella*, *Devriesia*, *Hortea*, *Penidiella*, *Phaeothecoidea*, *Pseodotaeniolina*, *Readeriella*, *Staninwardia*, *Stenella* and *Teratosphaeria* (Crous *et al.* 2009c). In this taxonomic rearrangement, many species of *Mycosphaerella* have been transferred to the closely related Teratosphaeriaceae and these include pathogens of *Eucalyptus* such as *T. cryptica*, *T. nubilosa,* T. *gauchensis* and *T. zuluensis* (Crous *et al.* 2007; Andjic *et al.* 2010).

Species of *Mycosphaerella* and *Teratosphaeria* have been identified from numerous hosts across a range of plant families (Crous and Groenewald 2005; Stukenbrock *et al.* 2007; Crous 2009). In South Africa, several Mycosphaerellaceae species infect *Eucalyptus* trees, namely *Mycosphaerella ellipsoidea*, *M. endophytica*, *M. marksii*, *M. pseudoendophytica*, *Pseudocercospora crystallina*, *P. epispermogonia*, *P. eucalyptorum*, *P. fori*, *P. irregulariramosa* and *P. natalensis* (Mycosphaerellaceae). Teratosphaeriaceae include *Teratosphaeria africana T. juvenis*, *T. nubilosa*, *T. odnowa*, *T. ovata*, *T. parva*, *T. suttonii*, *T. verrucosa*, *T. zuluensis* and *Readeriella stellenboschiana* (Crous and Wingfield

1996; Crous 1998; Hunter *et al.* 2004b; Hunter *et al.* 2011). *Mycosphaerella syzygii*, *M. marasasii* and *Pseudocercospora syzygiicola* have been found to be associated with the closely related native tree *Syzygium cordatum* (Sutton and Crous 1997; Crous 1999; Crous *et al.* 2001a).

In studies that aimed to characterise the endophytes of a *Eucalyptus grandis* x *E. camaldulensis* GC540 hybrid clone (Pillay 2012) and *S. cordatum* (**Chapter 2** of this thesis) with DNA barcodes, numerous isolates of species belonging to the Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae formed one of the dominant groups. However, sequences for the ITS region were unable to resolve the complex relationships within this group. The aim of the present study was to identify these species based on the most recently available multi-locus phylogenies.

2. Materials and Methods

2.1. Isolates used

Isolates from a single *Eucalyptus grandis* x *E. camaldulensis* hybrid clone (GC540) and a *Syzygium cordatum* tree, identified by Pillay (2012) and in **Chapter 2** of this thesis, as belonging to Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae were used in this study (Table 1). These are maintained in the culture collection (CMW) at the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa. Duplicates of isolates representing new host or geographical records or new taxa were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Herbarium specimens of these isolates have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

2.2. Multi-locus sequence analysis

Mycelium was scraped from the surface of cultures growing on 2 % Malt Extract Agar (MEA) (20 g MEA, 20 g Biolab agar, 1L distilled water; Biolab, Merck, Midrand, South Africa) and freeze dried for 24 hours. DNA was subsequently extracted using a modified CTAB method (Möller *et al.* 1992) as described in **Chapter 2**. The final DNA pellet was re-suspended in 50 µl double distilled SABAX water and incubated at 4 ºC for a few hours, preferably overnight (Möller *et al.* 1992). DNA concentrations of the samples were determined using a NanoDrop™ 1000 Spectrophotometer v. 3.7 (Thermo Fisher Scientific Inc.) and the DNA was diluted to 40 ng/µl.

The same PCR protocols used for the amplification of the ITS ribosomal DNA region (**Chapter 2**) were used for the amplification of the TEF-1α and a portion of the LSU gene regions. The primers used to amplify EF-1α were EF1-728F (5'-CAT CGA GAA GTT CGA GAA GG-3') and EF1-986R (5'-TAC TTG AAG GAA CCC TTA CC-3') (Carbone and Kohn 1999). LROR (5'-ACC CGC TGA ACT TAA GC-3') (Moncalvo *et al.* 1995) and LR7 (5'-TAC TAC CAC CAA GAT CT-3') (Vilgalys and Hester 1990) were used to amplify the LSU region. The cycling conditions for the ITS and EF-1α were identical, while those for LSU included: an initial degradation step of 96 ºC for 2 minutes, followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and elongation at 72 ºC for 1 minute with a final extension step at 72 ºC for 7 minutes. The PCR products were visualised on a 2 % agarose gel under ultraviolet (UV) light. The same primer set was used for the sequencing of TEF-1α. However, for the LSU region two additional internal primers were needed, namely LR3R (5'-GTC TTG AAA CAC GGA CC-3') (Vilgalys and Hester 1990) and LR5 (5'- TCC TGA GGG AAA CTT CG -3') (Rehner and Samuels 1995). The resultant sequences were analysed and contigs were assembled using CLC Main Workbench version 5.6 (CLC Bio A/S, Aarhus, Denmark).

For the generic placement of the isolates, the LSU sequences were aligned with LSU sequences of all current species belonging to Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae previously published (Crous *et al.* 2009b; Crous *et al.* 2009d) using MAFFT version 5.667 (Katoh *et al.* 2005) and using a dataset provided by Dr E. Groenewald (CBS). A neighbour-joining tree, with complete deletion, was produced in MEGA version 4.0 (Tamura *et al.* 2007) to determine the generic placement of the isolates. Due to the large size of the dataset, only the species representing the nearest neighbours to the isolates of unknown identity were kept for further analyses (data not shown). ITS and TEF-1α sequences of these nearest neighbours were obtained from the GenBank database housed at the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.hih.gov) and included in additional datasets. The sequences for the three gene regions were aligned using MAFFT version 5.667.

Parsimony trees were constructed using PAUP version 4.0 b10 (Swofford 2003). The heuristic search function with random sequence additions (100) and Tree Bisection Reconnection (TBR) algorithm was used to obtain the equally most parsimonious trees. All uninformative characters, including gaps and missing data, were excluded and characters were reweighted according to the consistency index (CI). Bootstrap values were calculated after 1 000 replicates to determine the level of branch support (Felsenstein 1985). Tree length (TL), consistency index (CI), retention index (RI) and the homoplasy index (HI) were all calculated (Table 2) to assess the trees for signal, noise and reliability (Hillis and Huelsenbeck 1992). A partition homogeneity test (PHT) was conducted and consisted of 1 000 replicates to determine if the ITS, LSU and TEF-1α datasets could be combined (Farris *et al.* 1994). The trees were rooted to *Neofusicoccum ribis* and *Phaeobotryosphaeria visci* as they are sister taxa to *Mycosphaerella* in the Dothideales (Maxwell *et al.* 2005).

MrBayes version 3 was used for Bayesian analyses of the various datasets (Ronquist and Huelsenbeck 2003). MrModeltest version 2.2 was first used to determine the nucleotide substitution model (Table 2) that would best fit the individual datasets (Nylander 2004). The Markov Chain Monte Carlo (MCMC) analyses of six chains from random tree topology were done twice on 1 000 000 generations and trees were saved every 1 000 generations. Tracer version 1.4 was used to determine the posterior distributions (Rambaut and Drummond 2007). Posterior probabilities were calculated to determine the level of branch support.

2.3. Morphological comparisons

Based on the phylogenetic trees constructed, two putative new species in *Mycosphaerella* and *Cladosporium* were recognised. Cultures representing these species were incubated on 2 % MEA at 25 ºC under near UV light for one month. For growth studies, mycelial plugs (5 mm) of each culture were transferred onto 65 mm Petri dishes with four replicates each of 2 % MEA plates and oats agar (OA; 30 g oats boiled in 500 ml distilled water, strained, with addition of 20 g agar and made up to 1L). The cultures were incubated in the dark at intervals of 5 ºC from 10 ºC to 30 ºC and two perpendicular measurements were taken daily over a period of a month. The averages and standard deviations were calculated using Microsoft Office Excel 2007 and the colour charts of Rayner were used to assign colours to the cultures and fruiting bodies (Rayner 1970).

To induce sporulation, carnation leaf agar (water agar, 20 g agar; 1L distilled water; 3 sterile leaf pieces 1 cm x 1 cm per plate) was used to grow the cultures under near UV light at 25 ºC for one month. To characterise the morphology of the putative new species of *Mycosphaerella*, pycnidia including conidiophores, conidiogenous cells as well as conidia were crushed on microscope slides in 80 % lactic acid. For the putative new *Cladosporium* species, the conidiophores where mounted on microscope slides and in

order to observe the conidiogenous cells and conidia, the conidiophores were mounted on slides with 80 % lactic acid. Where possible, 30 measurements were made for both the length and width of the various structures. Images were captured on a HRC Axiocam digital camera using the Axiovision version 3.1 software (Carl Zeiss Ltd, Germany). Measurements were taken and calculated with a 95 % confidence level.

3. Results

3.1. Multi-locus sequence analysis

Sequence data were obtained for 40 isolates, of which 13 were from a healthy *Syzygium cordatum* tree and 27 from a single individual of a healthy *Eucalyptus grandis* x *E. camaldulensis* clone (Table 1). For each of these 40 isolates, sequence data were created for the ITS ribosomal DNA, LSU of the nuclear ribosomal DNA operon and the TEF-1α gene regions. All available reference sequences for each dataset were included in the analyses in order to verify the identity of the isolates in this study (Table 1).

Maximum parsimony and Bayesian analyses were done on the datasets individually as well as in combination, with appropriate models as determined by MrModeltest version 2.2 (Table 2). The maximum parsimony and Bayesian trees for each gene region and combined data had similar topologies although, for the more conserved gene regions, clades often collapsed. The PHT value for the combined datasets was $p < 0.001$, which indicated that the datasets were not congruent. However, the data were combined since no inconsistencies in the major clades were observed, other than those mentioned above, to illustrate the support for the congruent clades.

Three distinct groups of isolates were observed (Figure 1), namely Cladosporiaceae (100% bootstrap support), Teratosphaeriaceae (100% bootstrap support) and Mycosphaerellaceae (100% bootstrap support). The isolates further grouped into eight clades and these included *Pseudocercospora crystallina*, *Cladosporium delicatulum*,

Mycosphaerella vietnamensis, *M. marksii*, *Readeriella considenianae* and *Teratosphaeria zuluensis* (Figure 1). Of these species, *Pseudocercospora crystallina* was isolated from both trees, whilst *M. marksii*, *M. vietnamensis*, and *C. delicatulum* were isolated solely from *S. cordatum*. The rest of the isolates were from the *E. grandis* x *E. camaldulensis* clone. Two groups of isolates from the *Eucalyptus* clone did not group with any of the known taxa. One grouped separately from all isolates representing known species in the Cladosporiaceae. The other formed a distinct clade in the Mycosphaerellaceae, grouping within *Mycosphaerella sensu stricto* based on the overall LSU alignment, which included all known taxa (data not shown).

3.2. Taxonomy

Two apparently undescribed species of Cladosporiaceae and Mycosphaerellaceae were collected from the *E. grandis* x *E. camaldulensis* trees in Mtubatuba, KwaZulu Natal. The Cladosporiaceae species was represented by a single isolate, whilst the Mycosphaerellaceae species was represented by 11 isolates. Based on morphological characteristics that are used to identify species in the Cladosporiaceae, Mycosphaerellaceae and Teratosphaeriaceae, it was determined that these fungal species represented new taxa and are subsequently described below:

Cladosporium impi prov. nom., **Marsberg, Gryzenh. & M.J. Wingf., Mycobank XXX (Figure 2 A-D)**

Etymology: The name refers to Zulu warriors from KwaZulu Natal, the area where the fungus was first collected; KwaZulu literally means “place of the Zulu”.

Typus: **South Africa**: *KwaZulu Natal*, Mtubatuba E32’9”54.1; S28’29”53.0, leaves of *Eucalyptus grandis* x *E. camaldulensis* hybrid clone (GC540), M. Gryzenhout, April 2009 (PREM XXX – holotypus; living cultures ex-type CMW 37667 = CBS XXX).

Anamorph only, cladosporium-like, *conidiophores* olivaceous-grey, cylindrical, macronematous, solitary, unbranched, arise laterally, straight to flexuous, no nodules, 88.3 - 205.7 x 2.8 - 7.1 µm. *Conidiogenous cells* integrated, terminal and intercalary with 2 – 3 loci at apex. *Secondary conidia* pale brown, ovoid to obovoid, (6.6-) 10.4 - 13.6 (-23.9) x (3.1-) 4.9 - 6.4 (-11.4) µm, aseptate with thickened hilia. *Conidia* pale brown, in branched chains branching in all directions, fusiform to ellipsoidal, (3.0-) 4.7 - 5.1 (-6.7) x (2.3-) 3.1 - 3.4 (-5.0) µm, thick-walled, aseptate with thickened hilium, conidia become conidiogenous cells.

Cultures: Colonies slow growing, 41 mm in diam on MEA and 48 mm in diam on OA after growth for one month at 25 ºC in the dark. On MEA, culture had even growth with a greenish olivaceous and honey surface, reverse greenish olivaceous and honey, mycelia dense, superficial as well as aerial, flat to raised with a felty to velvety texture, margins smooth. On OA, culture grey olivaceous on the surface and greenish olivaceous on the reverse, mycelia dense, raised and superficial with felt-like texture, smooth margins.

Optimum growth temperature 25 ºC (Figure 3).

***Mycosphaerella picanini prov. nom.,* Marsberg, Gryzenh. & M.J. Wingf., Mycobank XXX (Figure 2 E-G)**

Etymology: The name refers to a small Zulu child, in reference to the small size of the conidia and because this species was isolated from the KwaZulu Natal province.

Typus: **South Africa**: *KwaZulu Natal*, Mtubatuba E32’9”54.1; S28’29”53.0, leaves of *Eucalyptus grandis* x *E. camaldulensis* hybrid clone (GC540), M. Gryzenhout, April 2009 (PREM XXX – holotypus; living cultures ex-type CMW 37683 = CBS XXX).

Teleomorph absent. Anamorph colletogloeopsis-like with black pycnidia. *Conidiogenous cells* hyaline, phialidic, 5.4 - 7.0 x 1.7 - 2.3 µm. *Conidia* hyaline, solitary,

reniform, allantoid, ovoid and obovoid, (4.0-) 4.9 - 5.3 (-7.5) x (1.5-) 2.2 - 2.4 (-3.3) µm, indistinctly and rarely 1 septate with a thickened hilum.

Cultures: Colonies slow growing, 23 mm in diam on MEA and 32 mm in diam on OA after growth for one month at 25 ºC in the dark. On MEA, surface saffron in the centre with isabelline to buff margins, reverse rosy buff with isabelline to buff margins, mycelia dense, superficial and flat with velvety texture, margins undulated to radially restricted with lobate edges. On OA, surface hyaline with greenish olivaceous margins, reverse hyaline with olivaceous buff margins, mycelia dense, immersed, flat and velvety, margins radially restricted with lobate edges.

Optimum growth temperature 25 ºC (Figure 3).

4. Discussion

This study identified species of Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae that occurred commonly as endophytes on a healthy *Syzygium cordatum* and *Eucalyptus grandis x E. camaldulensis* hybrid clone (GC540) in South Africa by using multi-locus sequence analysis. A previous study (**Chapter 2**) showed that these groups represented 6.4 % of the endophytes isolated from *S. cordatum*. The results of the present study showed that they represent eight species, two of which are putative new species. These included *Mycosphaerella marksii*, *M. vietnamensis* and *Cladosporium delicatulum* from *Syzygium cordatum*, *Teratosphaeria zuluensis*, *Readeriella considenianae*, *C. impi prov. nom.* and *M. picanini prov. nom.* from the *Eucalyptus* GC540 clone and *Pseudocercospora crytallina* from both trees. *Cladosporium delicatulum*, *M. vietnamensis* and *R. considenianae* are here reported from South Africa for the first time, while it is also the first time that *P. crystallina*, *C. delicatulum*, *M.* and *M. marksii* have been found on a host other than *Eucalyptus*.

Teratosphaeria zuluensis was isolated from the leaves of the *Eucalyptus* GC540 clone. This well-known and serious pathogen is associated with *E. grandis* clones in South Africa (Wingfield *et al.* 1996; Cortinas *et al.* 2010) and it is thought to be a native pathogen that has adapted to infect the non-native *Eucalyptus* species. As a pathogen, *T. zuluensis* causes cankers on the stems and branches of *Eucalyptus* trees (Wingfield *et al.* 1996; van Zyl *et al.* 2002). In this study, *T. zuluensis* was a common endophyte of leaves. This discovery is interesting as symptoms of the pathogen have never been found on leaves, but they are common on green stem tissue, which is morphologically similar to leaf tissue. Whether the fungus can act as a pathogen of leaves is unknown, but given the intensity of surveys conducted in *Eucalyptus* plantations that have the canker disease, this seems unlikely. The role of *T. zuluensis* on leaves is curious and deserves further study.

Pseudocercospora crystallina was found in the leaves of both *S. cordatum* and the *Eucalyptus* GC540 clone. This fungus is known to occur on *E. bicostata* as well as on *Eucalyptus grandis* x *E. camaldulensis* GC540 in South Africa (Crous and Wingfield 1996). *Pseudocercospora crystallina* was previously known only from South Africa, but has since been found to occur on *Eucalyptus* in China (Burgess *et al.* 2007). It was interesting to find the fungus on a native South African tree and it suggests that it has the ability to cross-infect to hosts other than *Eucalyptus* spp. Given the occurrence of the fungus in China, it seems unlikely that it is native to South Africa. It can thus be considered an alien invasive of *S. cordatum*, although there is no indication that it is causing disease on this native tree.

Mycosphaerella marksii was identified from the leaves of *S. cordatum*, but not from the leaves of the *Eucalyptus* GC540 clone sampled in this study. *Mycospherella marksii* is, however, known to occur on many *Eucalyptus* species in Australia, China, Ethiopia, Indonesia, Madagascar, Portugal, South Africa and Uruguay, but is not considered to be an important pathogen (Crous and Wingfield 1996; Crous 1998; Hunter *et al.* 2004b;

Burgess *et al.* 2007; Hunter *et al.* 2011). This is thus the first report that *M. marksii* occurs on *S. cordatum* in South Africa and is another example of cross-infection.

Readeriella considenianae was isolated from the leaves of the *Eucalyptus* clone, *C. delicatulum* and *M. vietnamensis* were isolated from the leaves of *S. cordatum*. These species have not been reported from South Africa before. *Readeriella considenianae* has recently been found to occur on *E. considenianae* in Australia, but is not an important pathogen (Summerell *et al.* 2006; Taylor *et al.* 2012). *Mycosphaerella vietnamensis* is known to occur on *E. camaldulensis* and *Eucalyptus grandis* in Vietnam (Burgess *et al.* 2007). In this study it was isolated from *Syzygium cordatum*, and it would thus be of interest to determine if it also occurs on *Eucalyptus* spp. in South Africa.

Isolates of *C. delicatulum* have been collected across Asia, Europe and North and South America from indoor air to dust, seaweed and various plants and is known to be a saprophyte of leaves, fruits, stems and tubers or as secondary invaders in lesions formed by other pathogenic fungi (Bensch *et al.* 2010). This is the first report of *Cladosporium delicatulum* in South Africa.

Two new species were described in this study. *Cladosporium impi* and *M. picanini* were both isolated from the leaves of the *Eucalyptus* GC540 clone. These species add to the rich diversity of species in *Mycosphaerella* and *Cladosporium* that are already found in South Africa on numerous hosts (Hunter *et al.* 2004a; Hunter *et al.* 2004b; Crous *et al.* 2006a; Bensch *et al.* 2010; Hunter *et al.* 2011). The discovery of the two new species from *Eucalyptus* GC540 was not surprising, as *Eucalyptus* have a megadiverse fungal diversity and these trees have not been fully sampled in South Africa.

The isolates characterised in this study were derived from a single *S. cordatum* and a single *Eucalyptus* tree. This limited sampling in terms of the host, however, revealed a number of new host and geographical reports and cross-infectivity between these two tree

species. The overall results illustrate that there are many more fungi present in well-studied trees, such as *Eucalyptus*, and that their patterns of occurrence remain poorly understood.

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Table 1: Details of isolates which were used in the phylogenetic analyses.

Species	Accession		Host	Country	Genbank Accession		
	CMW [a]	Other [b]			ITS	LSU	TEF-1α
Cladosporium delicatulum	xxx14		*Syzygium cordatum*	South Africa	JQ732934	JQ732983	JQ733030
	xxx15			South Africa	JQ732935	JQ732984	JQ733031
	37700			South Africa	JQ732936	JQ732985	JQ733032
Cladosporium impi	37667		*Eucalyptus grandis* x *E. camaldulensis*	South Africa	JQ732888	JQ732937	JQ732986
Mycosphaerella marksii	37697		*S. cordatum*	South Africa	JQ732925	JQ732974	JQ733021
	xxxx6			South Africa	JQ732926	JQ732975	JQ733022
	xxxx9			South Africa	JQ732929	JQ732978	JQ733025
	xxx10			South Africa	JQ732930	JQ732979	JQ733026
	xxx11			South Africa	JQ732931	JQ732980	JQ733026
Mycosphaerella picanini	37673		*E. grandis* x *E. camaldulensis*	South Africa	JQ732895	JQ732944	JQ732993
	37677			South Africa	JQ732900	JQ732949	JQ732998
	37681			South Africa	JQ732904	JQ732953	JQ733002
	37682			South Africa	JQ732905	JQ732954	JQ733003
	37683			South Africa	JQ732906	JQ732955	JQ733004
	37684			South Africa	JQ732907	JQ732956	JQ733005
	37685			South Africa	JQ732908	JQ732957	JQ733006
	37686			South Africa	JQ732909	JQ732958	JQ733007

	xxx39		South Africa	JQ732914	JQ732963	JQ733011
	37689		South Africa	JQ732916	JQ732965	JQ733013
	xxx44		South Africa	JQ732918	JQ732967	JQ733015
Mycosphaerella vietnamensis	xxxx2	*S. cordatum*	South Africa	JQ732922	JQ732971	JQ733018
	37695		South Africa	JQ732923	JQ732972	JQ733019
	37696		South Africa	JQ732924	JQ732973	JQ733020
Pseudocercospora crystallina	xxx36	*E. grandis* x *E. camaldulensis*	South Africa	JQ732911	JQ732960	JQ733008
	xxxx7	*S. cordatum*	South Africa	JQ732927	JQ732976	JQ733023
	37698	*S. cordatum*	South Africa	JQ732932	JQ732981	JQ733028
Readeriella considenianae	37671	*E. grandis* x *E. camaldulensis*	South Africa	JQ732893	JQ732942	JQ732990
	37674		South Africa	JQ732896	JQ732945	JQ732994
	37675		South Africa	JQ732897	JQ732946	JQ732995
	xxx21		South Africa	JQ732898	JQ732947	JQ732996
	37676		South Africa	JQ732899	JQ732948	JQ732997
	37678		South Africa	JQ732901	JQ732950	JQ732999
	37680		South Africa	JQ732903	JQ732952	JQ733001
Teratosphaeria zuluensis	37669	*E. grandis* x *E. camaldulensis*	South Africa	JQ732891	JQ732940	JQ732988
	37670		South Africa	JQ732892	JQ732941	JQ732989

	37672			South Africa	JQ732894	JQ732943	JQ732992
	37679			South Africa	JQ732902	JQ732951	JQ733000
	37690			South Africa	JQ732915	JQ732964	JQ733012
	xxx43			South Africa	JQ732917	JQ732966	JQ733014
	37693			South Africa	JQ732920	JQ732969	JQ733016
Cladosporium cladosporiodes	-	AMAASA	-	India	JN618353	-	-
	-	CBS 401.80	*Triticum aestivum*	Netherlands	-	GU214409	-
	-	CPC 18230	*Phaenocoma prolifera*	South Africa	-	-	JF499872
Cladosporium funiculosum	-	CBS 122128	*Ficus carica*	Japan	HM148093	-	HM148337
	-	CBS 122129	*Vigna umbellata*	Japan	HM148094	AY342129	HM148338
Cladosporium oxysporum	-	CBS 126351	*Soil*	China	HM148119	AY342116	HM148363
	-	CBS 125991	*Indoor air*	Venezuela	HM148118	-	HM148362
Cladosporium uredinicola	-	NMG 15	Insect gallery	China	HM776420	-	-
		CPC 5390	*Quercus nigra*	U.S.A	-	EU019264	HM148467
	-	G6M-44	Liquor	China	JN227057	-	-
Cladosporium vignae	-	CBS 121.25	*Vigna unguiculata*	U.S.A	HM148227	AY342132	HM148473
	-	ATCC 90242	Indoor air	U.S.A	AY361998	-	-
Mycosphaerella ellipsoidea	-	CBS 110843	*Eucalyptus* sp.	South Africa	AY725545	-	-
	4934	-	*Eucalyptus* sp.	South Africa	-	DQ246253	DQ235129
	-	-	-	China	FJ490753	-	-
	-	CBS 110843	*Eucalyptus* sp.	South Africa	-	GQ852602	-

	5166	-	*Eucalyptus* sp.	South Africa	-	-	DQ235127
Mycosphaerella endophytica	-	CBS 114662	*Eucalyptus* sp.	South Africa	DQ302953	GU214435	-
	5225	-	*Eucalyptus* sp.	South Africa	-	-	DQ235128
	14912	CBS 111519	*Eucalyptus* sp.	South Africa	DQ267579	-	DQ235131
Mycosphaerella marksii	14781	CBS 682.95	*E. grandis*	South Africa	DQ267587	DQ246249	DQ235133
	5230	-	*E. botryoides*	Australia	DQ267588	DQ246246	DQ235135
Mycosphaerella vietnamensis	23442	-	*E. camaldulensis*	Vietnam	DQ632678	EU882135	-
	-	-	*Eucalyptus* sp.	Thailand			
	23441	CBS 119974	*E. grandis*	Vietnam	DQ632675	JF700944	-
Pseudocercospora crystallina	3042	-	*E. bicostata*	South Africa	DQ267578	DQ204746	DQ211662
	22534	-					
	3033	CBS 681.95	*E. bicostata*	South Africa	DQ632681	DQ204747	DQ211663
Phaeophleospora eugeniicola	-	CPC 2558	*Eucalyptus* sp.	Brazil	FJ493191	FJ493209	-
	-	CPC 2557	*Eucalyptus* sp.	Brazil	FJ493190	-	-
	-	WM 05.11	-	Australia	EF568045	-	-
	-	CPC 11609	*Musa* sp.	India	-	-	EF679431
	-	CBS 109.21	*Hedera helix*	India	-	EU019262	-
Readeriella considenianae	-	-	*E. stellulata*	Australia	GQ852792	GQ852681	-
	-	-	*E. stellulata*	Australia	GQ852791	GQ852680	-
Teratosphaeria molleriana	4940	CBS 111164	*E. globulus*	Portugal	AF309620	DQ246220	DQ235104
	2734	CBS 111132	*E. globulus*	U.S.A	AF309619	DQ246223	DQ235105

Teratosphaeria nubilosa	**3282**	**CBS 116005**	***E. globulus***	**Australia**	**AF309618**	**DQ246228**	**-**
	-	**CBS 112972**	***E. nitens***	**South Africa**	**AY725564**	**EU019304**	**-**
	3282	**CBS 116005**	***E. globulus***	**Australia**			
Teratosphaeria zuluensis	**17320**	**-**	***E. grandis***	**Zambia**	**DQ240148**	**EU019296**	**DQ240206**
	-	**CBS 120301**	***E. grandis***	**South Africa**			
Neofusicoccum ribis	**7773**	**-**	***Ribis* sp.**	**U.S.A**	**DQ246604**	**DQ246263**	**DQ235142**
Phaeobotryosphaeria visci	**-**	**CBS 100163**	***Sphaeropsis visci***	**Germany**	**EU673324**	**DQ377870**	**EU673292**

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

[b] CBS = the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands; ATCC = American Type Culture Collection, George Mason University, Manassas, Virginia, USA

- = not available

Table 2: Statistics results for phylogenetic analyses

Maximum Parsimony

Dataset	Number of Taxa	Number of Excluded Characters	Number of Included Characters	Tree Number	Tree Length	CI	RI	HI
ITS	56	954	159	24	348	0.750	0.952	0.250
LSU	81	364	681	648	1 165	0.750	0.955	0.250
TEF-1α	55	22	288	1 134	1 208	0.617	0.918	0.383
Combined	55	951	743	720	2 217	0.646	0.927	0.354

MrBayes

Dataset	Model	Preset state freqpr	NST	Rates	Burnin
ITS	GTR+I+G	(1, 1, 1, 1)	6	invgamma	1 000
LSU	GTR+G	(1, 1, 1, 1)	6	gamma	1 000
TEF-1α	GTR+G	(1, 1, 1, 1)	6	gamma	1 000
Combined	GTR+I+G	(1, 1, 1, 1)	6	invgamma	1 000

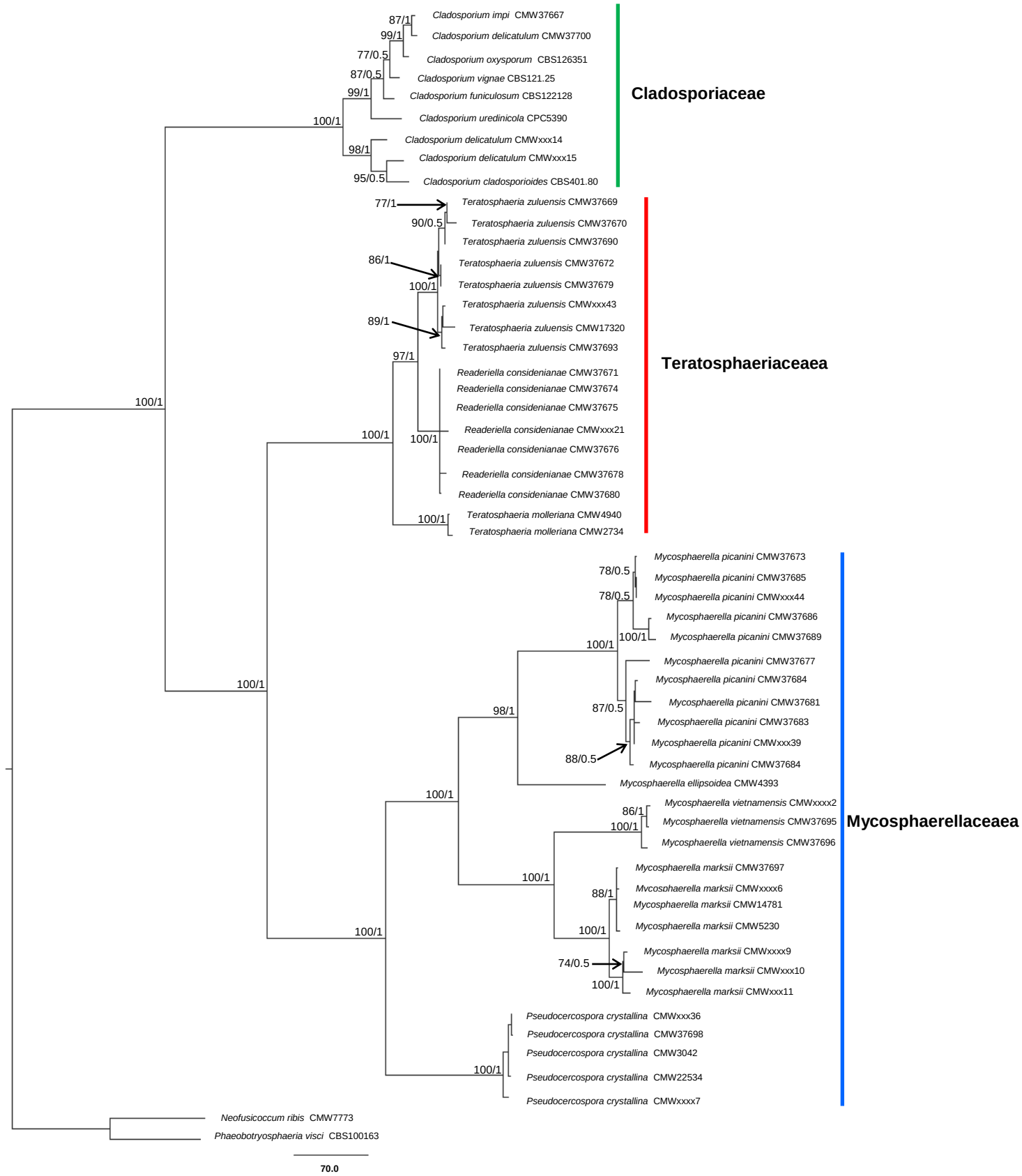

Figure 1: One of the equally most parsimonious trees obtained from a heuristic search of the combined ITS, LSU and TEF-1α datasets. Bootstrap support values obtained from 1 000 replicates along with posterior probabilities are shown at the nodes. The tree was rooted with *Neofusicoccum ribis* and *Phaeobotryosphaeria visci*. Tree length = 2 217, CI = 0.646, RI = 0.927 and HI = 0.354.

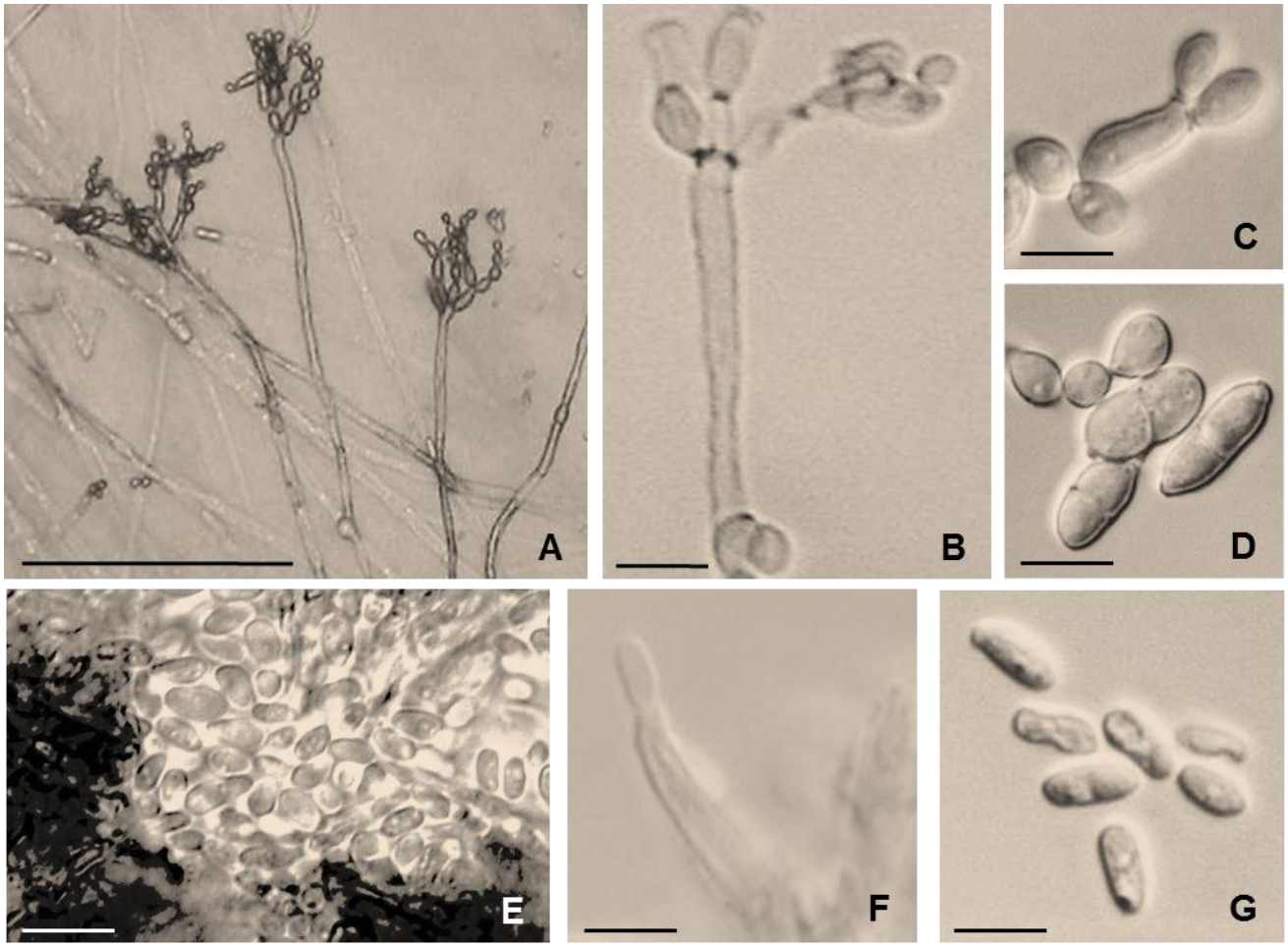

Figure 2: ***Cladosporium impi*** **A-D**. **A-B** Branched conidiophore; **C** Chains of conidia; **D** Conidia. ***Mycosphaerella picanini*** **E-G**. **E** Conidia being released from pycnidium; **F** Phialide conidiogenous cell; **G** Conidia. **Scale bars** A=100 µm; B=20 µm; C-G=5 µm.

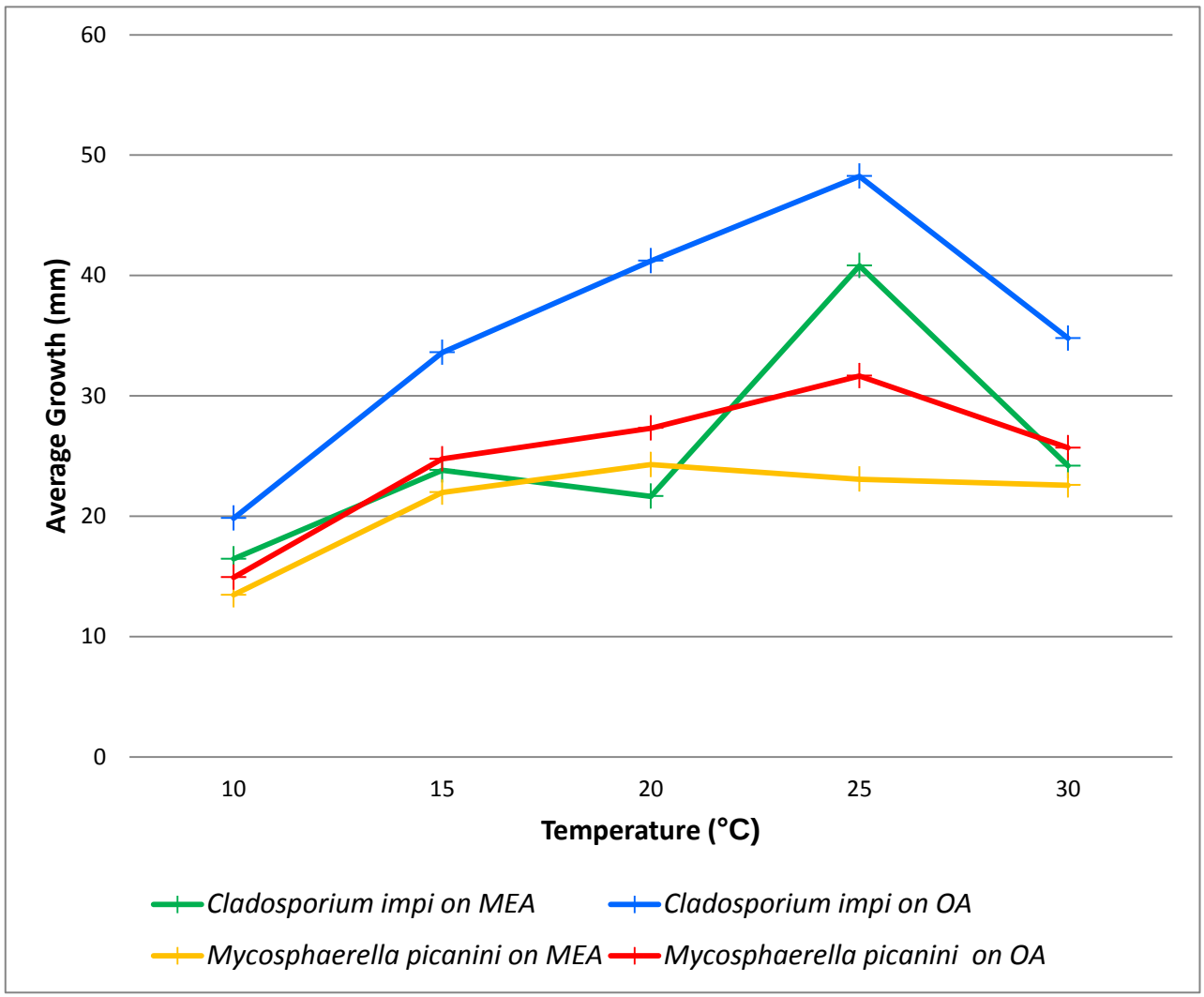

Figure 3: Graph of the optimum growth temperatures of *Cladosporium impi* and *Mycosphaerella picanini* on Malt Extract Agar (MEA) and Oats Agar (OA) after 1 month in the dark.

SUMMARY

Endophytes are organisms that are capable of colonising plant tissues without causing any visible signs of disease. Evidence, however, shows that endophytes have evolved from pathogenic fungi as many endophytes are latent pathogens or saprophytes that only sporulate when their host is stressed or dying. The ecological role and basis for the interaction between trees and their diverse assemblage of endophytes remain unclear. This is especially true in South Africa where very few studies have been done on fungal biodiversity as taxonomic expertise is scarce. The aim of this study was to, therefore, determine the endophytic diversity associated with *Syzygium cordatum* and it was expected that all isolated fungi would be native to South Africa as the host is a native tree found in the KwaZulu Natal province.

Chapter 1 of this thesis aimed to understand the complex theory surrounding fungal endophytes by reviewing available literature. By doing so, the complexity of the endophyte-host interaction, mode of infection, the influence that endophytes have on their hosts, the co-evolution of their life-history traits and why endophytes are important in their environments were gauged. It is, however, clear that the knowledge pertaining to the diversity, ecology and evolution of endophytes is generally incomplete, despite the common occurrence of these diverse organisms.

In **Chapter 2** of this study, the combined use of isolate-based and culture-independent environmental barcoding, sampled the endophytic diversity associated with *S. cordatum*. Isolate-based barcoding identified 250 isolates which represented 13 orders, 22 families and 42 species groups. This is contrasted with the culture-independent

method of environmental barcoding in which 15 orders, 20 families and 92 MOTUs were detected with 454 GS FLX Titanium pyrosequencing analysis. In the culture-based approach a *Pestalotiopsis* species was dominantly isolated while a diverse array of Mycosphaerellaceae species were the dominant sequences generated with pyrosequencing.

In **Chapter 3** of this study, *Mycosphaerella marksii*, *M. vietnamensis* and *Cladosporium delicatulum* were isolated from *S. cordatum*, whilst *Teratosphaeria zuluensis*, *Readeriella considenianae* and the two new species *C. impi* and *M. picanini* were isolated from the *Eucalyptus grandis* x *E. camaldulensis* clone and *Pseudocercospora crystallina* was isolated from both trees. *Cladosporium delicatulum*, *M, vietnamensis*, and *R. considenianae* were reported in South Africa for the first time and new host reports in the case of *P. crystallina*, *C. delicatulum*, *Mycosphaerella vietnamensis* and *M. marksii* were reported from *S. cordatum*. Despite the limited number of trees that were sampled, some light was shed on the diversity of Mycosphaerellaceae, Teratosphaeriaceae and Cladosporiaceae that are associated as endophytes with Myrtaceae species in South Africa.

This study provides a stepping stone towards understanding fungal endophyte diversity and contributes to the limited knowledge available on fungi in South Africa. It also highlights the need for verified databases which will be constructed with isolate-based barcoding techniques. Once a reliable database is established, environmental barcoding can be used to explore fungal diversity in a reliable, high-throughput manner.