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Characterization of *Lecanosticta* and *Lophodermium* species on non-native pines in the Southern Hemisphere

by

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DECLARATION OF ORIGINALITY

I, Ms Cheyenne Amor Theron, declare that the dissertation which I hereby submit for the degree ***Magister of Scientiae*** **(Plant Pathology)** 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.

CHEYENNE AMOR THERON

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DATE

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PREFACE

Many countries have established commercial plantations of *Pinus* spp. to accommodate a growing demand for wood and timber products. In the Southern Hemisphere, where *Pinus* spp. are non-native, establishment of these plantations commenced in the mid-19th century. Several different species were introduced and tested on different sites, and some have more recently been selected for breeding and the establishment of hybrid clones. This has resulted in the considerable movement of seed and other forms of germplasm across different continents and concomitantly microbial pathogens have also been introduced into new environments. Several of these organisms include important pathogens and consequently there has been an emergence of disease outbreaks that, in some cases, have severely impacted the profitability of commercial plantations in the Southern Hemisphere. There are also many pathogens of *Pinus* spp. that could be accidentally introduced in the future, and these pose a significant potential threat to the sustainability of plantation forestry in the Southern Hemisphere.

An important threat to plantation grown non-native *Pinus* spp. is the disease Brown spot needle blight (BSNB) caused by *Lecanosticta acicola*. This disease is widespread in the Northern Hemisphere, affecting native and planted *Pinus* spp. In the Southern Hemisphere, this disease has been reported only in Colombia and apart from a severe outbreak in the 1980s, has not warranted concern in that country or the region in general. Recently, however, several other species of *Lecanosticta* have been described from *Pinus* spp. native to Mesoamerica and this has led to the hypothesis that this region may be the centre of origin of these fungal species. Mesoamerican *Pinus* spp. and their hybrids are now being increasingly used for commercial forestry in many Southern Hemisphere countries and extreme caution must be taken not to accidentally introduce these *Lecanosticta* species.

Approximately 38 species of *Lophodermium* have been described infecting needles and cones on *Pinus* spp. Apart from *L. seditiosum*, the causal agent of Lophodermium needle cast, all other species are recognized as endophytes and not primary pathogens of healthy trees. Phylogenetic analyses have recently revealed that several

of the *Lophodermium* spp. identified, historically based on morphology, may contain cryptic species. In addition, recent studies on the endophytic communities of native and planted *Pinus* spp. in the Northern Hemisphere have also revealed unidentified species of *Lophodermium*. Most historical reports of *Lophodermium* spp. on non-native *Pinus* spp. introduced into the Southern Hemisphere have relied solely on morphological characteristics for identification and it is not known whether any new incursions have recently occurred.

This dissertation focusses on what is known regarding the pine needle diseases that have been reported affecting non-native *Pinus* spp. commercially grown in the Southern Hemisphere, especially pertaining to the genera *Lecanosticta* and *Lophodermium*. Although not strictly in the Southern Hemisphere, Colombia was included in this study due to the non-native nature of the *Pinus* spp. commercially grown in this country and that has a similar history of *Pinus* forestry as other countries in the Southern Hemisphere. This dissertation includes a literature review and two independent research chapters. The second chapter was published in the journal of *Plant Disease* and for uniformity, the literature review and third chapter are presented following the style of that journal.

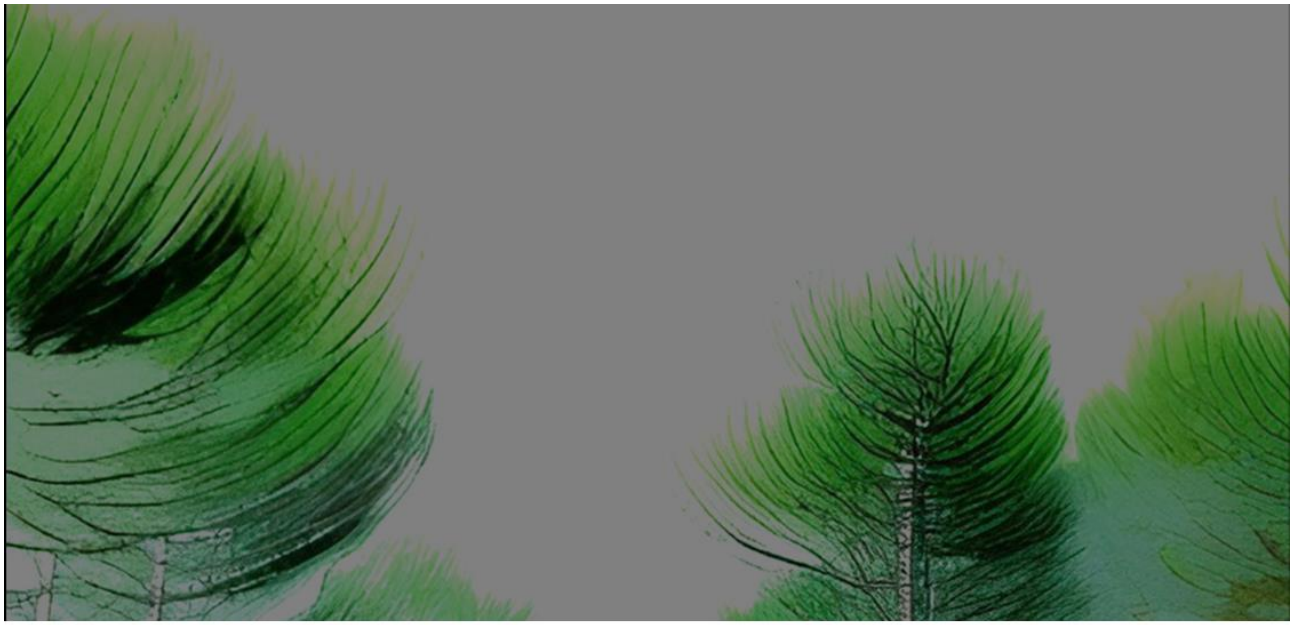
The first chapter of this dissertation presents a review of the literature on pine needle diseases that have had an impact on commercial forestry in the Southern Hemisphere in the past, or that are considered an emerging threat to pine plantations in the future. The review aims to condense the large volumes of work done on these diseases by providing a short description of the taxonomic history, distribution, host range and biology and symptoms of each of the causal agents. In addition, information is also provided for those diseases where the identity of causal agents was confirmed using DNA sequence-based techniques.

Since 2018, symptoms resembling BSNB began to occur on Mesoamerican *Pinus* spp. commercially grown in Colombia and for which the causal agent was suspected to be a species of *Lecanosticta*. Several past reports of *L. acicola* in Colombia were based on morphological descriptions and the only confirmation of the identity of this pathogen using DNA-based sequencing techniques was for a small number of isolates from *Pinus caribaea.* Given that several species of *Lecanosticta* have recently been

reported from *Pinus* spp. native to Mesoamerica, the aim of this chapter was to isolate and determine the identity of the causal agent of the disease outbreak employing both a DNA sequence-based and morphological approach.

The aim of the third chapter of this dissertation was to determine the diversity and distribution of *Lophodermium* spp. from the Southern Hemisphere. Most of the reports of *Lophodermium* spp. in the Southern Hemisphere, including the suggested presence of the pathogenic species *L. seditiosum* in Chile and South Africa, have relied solely on morphological characteristics for identification. A small number of isolates from Australia and New Zealand, identified using DNA sequence data, revealed the presence of mainly non-pathogenic *Lophodermium* spp. being present in these countries. This, and the fact that there has never been evidence of Lophodermium needle cast in Southern Hemisphere *Pinus* plantations, calls into question the reliability of past reports of these fungi. The studies in this chapter aimed to confirm the identity of *Lophodermium* spp. collected from non-native *Pinus* spp. planted in the Southern Hemisphere (including Colombia). Morphological and DNA sequence comparisons were used to determine the identity of a large collection of isolates, including herbarium specimens, from Australia, Colombia, Chile, New Zealand and South Africa.

Cover pages at the beginning of each chapter represent a cartoon of images collected during each chapter created in the Dream by Wombo platform (http://wombo.art).



CHAPTER 1

The distribution and molecular confirmation of important foliar pathogens of *Pinus* spp. in the Southern Hemisphere

ABSTRACT

Commercial plantations of non-native *Pinus* have been established in the Southern Hemisphere for almost two centuries. The productivity of these plantations has been increasingly threatened by microbial pathogens. Infection by these pathogens can lead to defoliation, stunted growth and, in severe cases, tree death. Accurate identification of needle pathogens is an important step towards disease monitoring and control. Although morphological and ecological techniques have proven useful for this purpose in the past, the development of DNA based phylogenetic, and population genetic analyses has substantially improved the ability to identify and better understand these pathogens. In this review, eight foliar diseases that have either severely affected or that pose a threat to non-native *Pinus* plantations in the Southern Hemisphere, are considered. They include diseases caused by the important pathogens such as *Diplodia sapinea, Dothistroma septosporum* and *Phytophthora* spp. Information is provided on the taxonomic history, distribution in the Southern Hemisphere, biology, and symptoms for each of the pathogens. This review highlights the fact that of the 96 reports of these pathogens in the Southern Hemisphere, the causal agents have only been confirmed using molecular techniques in 35 cases. Commercially managed plantations of non-native *Pinus* spp. will remain economically important to Southern Hemisphere countries well into the future and molecular techniques to identify and track the movement of needle blight pathogens will become increasingly important.

1. INTRODUCTION

Forest trees belonging to the genus *Pinus* L. are of great importance, playing a role in ecosystem functions and for commercial plantation purposes (Richardson, 1998; Boyd et al., 2013). Over the past two centuries, pine trees have been extensively planted as non-natives in the Southern Hemisphere, with *P. radiata* D. Don one of the first species utilised for this purpose (Legat, 1930). Interestingly, plantations of *P. radiata* in Chile, New Zealand and Australia account for over 90% of the world's radiata pine plantations (Mead, 2013).

Species of *Pinus* can grow in a wide variety of different climatic conditions. Consequently, in the early 1900s trials were established in many countries of the Southern Hemisphere to determine the suitability of different species to the specific climatic conditions in these regions (Legat, 1930; Turner, 1932; Rodger, 1946; Mead, 2013). From these results, desirable species were chosen, nurseries were established and extensive breeding programmes were initiated for tree improvement, including hybridization and large-scale vegetative propagation (Webb et al., 1984; Richardson, 1998; Mead, 2013). These plantations contribute to world trade in wood and wood products, including timber, pulp and paper.

Human globalization has increased rapidly since the 18th century and consequently the demand for wood and wood products was undertaken by many Southern Hemisphere countries (Lambin and Meyfroidt, 2011). However, in the last decade, with improvements in augmented trade and transport, the geographical barriers for organisms associated with *Pinus* spp. have also been overcome (Wingfield, 1999; Wingfield et al., 2001; Hulme, 2009). This includes the introduction of important fungal and oomycete pathogens, such as *Diplodia sapinea* (Fr.) Fuckel, and *Phytophthora pinifolia* Alv. Durán, Gryzenh. & M.J. Wingf*.,* respectively, into Southern Hemisphere plantations. Pathogens such as these are prominent agents of infectious diseases and pose a major economic and environmental risk to commercial forestry (Santini et al., 2013). It is, therefore, important that these pathogens are correctly identified and monitored, and that quarantine measures are implemented to prevent future introductions.

Historically, morphological and ecological studies have proven useful in identifying the causal agents of disease outbreak (Jayawardena et al., 2021). However, without the trained eye of an expert and the subjectivity associated with the choice of morphological features used for identification, such studies can result in

equivocal results (Bonants et al., 2010; Jayawardena et al., 2021). DNA sequence data, used in conjunction with morphological analyses, has become essential to accurately differentiate species, especially when the species have differences in host associations, geographical distribution and disease severity (Shivas and Cai, 2012; Reignoux et al., 2014; van der Nest et al., 2019b).

Molecular studies using informative genetic markers has also been useful in determining the origin and invasion pathways of plant pathogen species (McDonald and McDermott, 1993; Giraud et al., 2008; Grünwald and Goss, 2011). As an example, *Lecanosticta acicola* (Thüm.) Syd., the causal agent of brown spot needle blight (BSNB) is a serious pathogen of *Pinus* spp. across the Northern Hemisphere (Wakeley, 1970; Quaedvlieg et al., 2012; van der Nest et al., 2019a). Phylogenetic analyses of several gene regions showed the cryptic diversity of nine *Lecanosticta* species on native *Pinus* spp. in Mesoamerica and considered this region to be the centre of origin of the genus (Quaedvlieg et al., 2012; van der Nest et al., 2019b). Such information is important as many of the commercial plantations established in the Southern Hemisphere are comprised of Mesoamerican *Pinus* spp. and caution should be taken to prevent the introduction of these pathogens in the future.

There have been numerous reports of foliar fungi and pathogens on *Pinus* spp. in the Southern Hemisphere during the course of the last century. Several of these reports are found in obscure journals, and there has not been a reasonable review on the foliar pathogens that have had an impact on the pine plantation industry in the Southern Hemisphere in the past. In this review we focus on eight needle diseases and the pathogens that cause them, as examples of some of the most important threats to non-native pine plantations in the Southern Hemisphere. The choice of these pathogens is either based on the impact they have had in shaping commercial forestry in the past, or where they are considered a potential risk to *Pinus* plantations in the future. Information is also provided as to which pathogens have been identified using DNA sequence-based techniques. The distribution, host range, biology and symptoms are discussed for each pathogen. Furthermore, areas where additional research is required to determine the implications these pathogens may have on future commercial plantations are highlighted.

2. *PINUS* PLANTATIONS IN THE SOUTHERN HEMISPHERE

The genus *Pinus* is taxonomically large including over 110-120 extant species (Richardson, 1998; Eckert and Hall, 2006). Species belonging to this genus bear great ecological and economic importance. All but one species, *Pinus merkusii* Jungh. & de Vriese, is native to the Northern Hemisphere and they are distributed across a variety of climates from boreal to temperate and tropical. Two major sub-genera are accepted in *Pinus*: *Strobus*, containing haploxylon or soft wood species and *Pinus* containing diploxylon or hard wood species (Price et al., 1998). Pine trees can grow in disturbed areas and have a profound ability to grow in nutrient poor soils. Their ability to tolerate such conditions and produce a large volume of wood has led to the introduction of *Pinus* species for commercial plantation purposes into the Southern Hemisphere (Richardson et al., 1994; Richardson, 1998).

Commercial planting of *Pinus* in regions of the Southern Hemisphere commenced towards the middle of the 19th century. The first country to successfully propagate these trees was South Africa in 1825 when plantations of *Pinus pinaster* Ait. were established in Genadedal, Western Cape province for commercial purposes (Legat, 1930). Species trials showed that *P. radiata* was more favourable and this was the first case to exemplify the importance of matching the *Pinus* spp. with the specific site characteristics (Legat, 1930).

In Australia, the diverse attributes of the land and climate types led to the introduction of several species into the different regions. In South Australia, afforestation commenced in 1876 and *P. radiata* was the species of choice for forestry. Western Australia was rich with indigenous forest, but the demand for softwoods led to the commencement of afforestation in 1920 with *P. pinaster* that proved better suited to these areas (Rodger, 1946). In New Zealand, afforestation began in 1896 with *Pinus nigra* Arnold and *Pinus ponderosa* Lawson &. C. Lawson and later the more favourable *P. radiata* was recommended (Turner, 1932) .

In the twentieth century, plantations began to be established in regions of South America and areas of Africa beyond South Africa. The first successful plantations were established in Argentina in 1927 and consisted of *P. ponderosa* (Cozzo, 1987). The first species trials in Brazil commenced around 1936 with different European *Pinus* spp. The environmental conditions, however, showed that these species were unfavourable and in 1948 four species introduced from North America showed promise; *P. palustris* Mill., *P. echinata* Mill., *P. elliottii* Engelm. and *P. taeda* L.

(Baldanzi et al., 1974; Simberloff et al., 2010). In Eswatini (previously known as Swaziland), plantations established in 1950 consisted of similar North American species and *P. patula*, different from Zambia, where plantations established in the 1960s mainly consisted of the Asian *Pinus kesiya* Royle: Gordon, which was later replaced by the more productive *Pinus oocarpa* Schiede from Central America (Ivory, 1977; Evans, 1988). For many countries, such as those discussed above, their silvicultural history is expansively documented and can be accurately traced. Many such reports emphasize the trials of several different *Pinus* species that were tested, and the species selected are now known to form the foundation of many plantations areas.

The most prominent species currently planted in the Southern Hemisphere include: *Pinus caribeae* Morelet*, P. elliottii, P. patula, P. radiata* and *P. taeda* (Ugalde and Perez, 2001). As already illustrated, *P. radiata* is one of the most popular species in this region where it performs well in Mediterranean climates resembling its native range along the Californian Coast and Guadeloupe Island (Ugalde and Perez, 2001; Burdon et al., 2017). This is especially true in New Zealand and Australia where breeding trials are continuously performed to produce genetically improved planting lines (Burdon et al., 2008). Although North American and Eurasian *Pinus* spp. were widely planted in the past in countries such as South Africa etc., Mesoamerican *Pinus* spp. have become more popular due to their ability to colonize specific environmental niches (Hodge and Dvorak, 2012).

Pinus patula is a temperate to subtropical species, native to eastern and southern Mexico, which has become important in summer rainfall areas in South America and several regions of Africa (Kanzler et al., 2012). However, the superiority of *P. patula* as a choice species began to decline once foresters found that these trees are susceptible to various fungal diseases such as pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell and Diplodia tip blight, caused by *D. sapinea* (Gibson, 1970; Viljoen et al., 1994; Kanzler et al., 2012; Steenkamp et al., 2012). To overcome this constraint, planting stock of *Pinus tecunumanii* Eguiluz & J. P. Perry was chosen to establish plantations in tropical and subtropical areas (Barnes and Styles, 1983; Kanzler et al., 2012; Rodas and Wingfield, 2020). *Pinus tecunumanii* performs well in countries such as Brazil, Colombia and South Africa and has shown to outcompete other species such as *P. taeda* (Hodge and Dvorak, 2012; Rodas et al., 2016).

Barnes and Styles (1983) suggested that interspecific breeding of similar clones from Mesoamerica should be implemented to produce hybrids with superior qualities. Hybrids of *P. patula* and *P. tecunumanii* have, for example, shown increased growth, wood productivity and disease tolerance and may eventually replace pure *P. patula* stands in Colombia and Southern Africa (Hodge and Dvorak, 2012; Kanzler et al., 2012). *Pinus maximinoi* H. E. Moore, a common species in Mesoamerica, has also shown great potential and plantations of this species have increased in regions such as Brazil, Colombia and Mozambique (Hodge and Dvorak, 2012; Rodas et al., 2016). Breeding programmes have also shown that the climatic requirements of *P. maximinoi* can make it suitable for forestry in Kenya, South Africa and Tanzania (Hodge and Dvorak, 2012). Breeding and selection programs such as these will likely benefit from interchanging genetic material between different regions that produce hybrids with combined desirable traits (Hodge and Dvorak, 2012).

Initial trials to determine the suitability of a certain species of *Pinus* for a specific area was largely determined by abiotic factors such as the climatic conditions of the regions and the occurrence of natural disturbances such as fire or hailstorm events (Webb et al., 1984; Mead, 2013). Over the past two centuries, introduction of different *Pinus* spp. for site selection and breeding of intraspecific clones has resulted in the unprecedented movement of seed and germplasm across continents. However, as time has passed, damaging pathogens have appeared, causing disease problems and the structure, composition and community of the plantations were altered accordingly (Castello et al., 1995).

3. FUNGI AND OOMYCETES INFECTING NEEDLES OF *PINUS* SPP.

Fungi are a diverse group of organisms and previously it was estimated that 1.5 million species may exist (Hawksworth, 2001). Recent studies have led to the revision of this estimate and based on the recognition of cryptic and semi-cryptic species, the diversity is now estimated ranging between 2.2 to 3.8 million (Hawksworth and Lücking, 2017). An obscure “hot spot” that is assumed to be species rich, especially with undescribed fungi, is the phyllosphere of plants in tropical forests (Hawksworth and Rossman, 1997). Pines, especially those from the tropics and sub-tropics, are perennial trees that have a high proportion of recalcitrant chemicals in their needles, making it a suitable microhabitat for many fungal species. In this regard, a variety of

fungi colonize the phyllosphere of *Pinus* spp., including endophytes, saprophytes, and pathogens (Botella and Diez, 2011).

Endophytes are organisms that colonize the green tissue of the plant without causing disease (Sieber, 2007; Delaye et al., 2013). Numerous studies have considered the endophytic communities on *Pinus* and have shown that these fungi may have a mutualistic relationship with the trees. This relationship provides the trees with protection against herbivores or pathogens, tolerance to stress and improved growth (Carroll and Carroll, 1978; Schulz et al., 2002; Ganley et al., 2004; Botella and Diez, 2011). However, pathogens that have a latent phase may also be considered endophytic until the host or environment changes in favour of disease development (Sieber, 2007; Sun and Guo, 2012). Latent infections of *D. sapinea*, for example, are well-known in *P. patula* stands from South Africa, resulting in disease outbreaks after severe hailstorms (Flowers et al., 2001; Smith et al., 2002; Bihon et al., 2011a).

Many fungi do not cause serious disease in their native range and as such would be considered harmless endophytes. However, when these fungi are inadvertently introduced from their native ranges along with their *Pinus* hosts into the Southern Hemisphere, they can encounter genetically uniform plantations of susceptible hosts, resulting in disease outbreaks (Park, 2002; Ghelardini et al., 2016). Germplasm provides a common route of introduction of pathogens. In this regard, pathogens causing foliar diseases are amongst the most common organisms reported in non-native plantations due to their association with the seeds and plant debris (Wingfield et al., 2001; Liebhold et al., 2012).

Foliar diseases reduce the photosynthetic capability of the needles, reducing the tree vigour and, depending on the cohort of needles affected, eventually result in stunted growth and occasionally death (Cordell et al., 1989; Rossing et al., 1992; Manter et al., 2003). Many pathogens capable of infecting the foliage of *Pinus* spp. belong to the *Ascomycota*, although there are a few *Basidiomycota* rusts and recently, oomycete species belonging to the *Oomycota* that cause such problems (McKenzie, 1998; Durán et al., 2008; Bednářová et al., 2013; Dick et al., 2014; Hansen, 2015). Foliar pathogens of *Pinus* spp. mainly manifest as needle blight and needle cast diseases (Bednářová et al., 2013).

Determining the identity of the causal agent of a disease outbreak is important for biosecurity and control. Traditionally, fungal species were identified based on a few morphological characteristics and ecological features, which complicated this process

(Cai et al., 2011; Sun and Guo, 2012). However, the ability to identify and delimit fungal species has improved with the advent of molecular techniques (Bonants et al., 2010; Shivas and Cai, 2012; Luchi et al., 2020). As such, morphologically indistinguishable species have been found to contain cryptic taxa (Crous and Groenewald, 2005; Cai et al., 2011; Shivas and Cai, 2012). For example, Dothistroma needle blight (DNB) was thought to be caused by a single species of *Dothistroma* for many years. In 2004, Barnes et al. (2004) showed that isolates responsible for the disease fall within separate phylogenetic lineages and actually represent two distinct species now known as *D. septosporum* (Dorog.) Morelet and *D. pini* Hulbary. The importance of being able to distinguish between the cryptic species of *Dothistroma* is that these species can differ significantly in host range, geographic distribution and ability to cause disease (Drenkhan et al., 2016). Therefore, identification based on molecular techniques is a reliable and robust method to correctly identify and detect important plant pathogenic organisms.

In the last century, there have been several reports of emerging foliar fungal and oomycete pathogens on *Pinus* spp. in the Southern Hemisphere. The outbreaks caused by some of these, such as *D. septosporum* and *D. sapinea*, have been well studied and documented (Burgess et al., 2004a; Bihon et al., 2012a; Barnes et al., 2014; Drenkhan et al., 2016). However, many of these reports from the Southern Hemisphere have not included identifications using DNA sequence data and may need to be re-examined. The objective of this review is consequently to consolidate all available information for the most prominent foliar pathogens that have been reported on *Pinus* spp. in the Southern Hemisphere and including those that could represent a potential future threat for forestry. An important outcome of this study is a table indicating the earliest date when each of these pathogens were reported on non-native *Pinus* spp. in countries from the Southern Hemisphere and whether these reports have been verified using DNA sequence data.

4. FOLIAR PATHOGENS REPORTED FROM THE SOUTHERN HEMISPHERE

4.1. Brown Needle Disease

4.1.1 Introduction

Brown needle disease, also known as Cercospora needle blight, was first discovered in Japan in 1917 severely affecting *P. pinaster, Pinus densiflora* Siebold & Zucc. and *Pinus thunbergii* Parlatore seedlings (Ito, 1972). Since its discovery, the

disease has become a major problem in native and non-native *Pinus* plantations, especially in the late nursery stage (Sullivan, 2010). It is caused by the ascomycete fungus, *Pseudocercospora pini-densiflorae* (Hori & Nambu) Deighton, which mainly occurs in tropical and subtropical countries where *Pinus* spp. are found (Ivory, 1994). In the 1960s, young plantations of *P. radiata* in Tanzania were severely impacted by this disease and in extreme cases young trees were killed (Mulder and Gibson, 1972; Suto, 1979).

4.1.2 Taxonomy

The causal agent of Brown needle disease was isolated from *P. densiflora* seedlings and subsequently identified as the asexual state of *Cercospora pini-densiflorae* Hori & Nambu (Ito, 1972). The sexual state of this fungus was later found on native pine plantations in Central America and *P. radiata* in Tanzania and named *Mycosphaerella gibsonii* H.C. Evans (Evans, 1984). After taxonomic revision of the genus, the pathogen was reclassified as *Pseudocercospora pini-densiflorae* (Hori & Nambu) Deighton (Deighton, 1987), and this is currently the recognized name of the pathogen responsible for Brown needle disease.

4.1.3 Distribution in the Southern Hemisphere

Brown needle disease remained confined to the Asian continent until the 1960s, after which it was reported in parts of East and Central Africa and Southeast Asia (Gill, 1963; Ito, 1972; Gibson, 1979). Since then, *P. pini-densiflorae* has been reported in nine countries from the Southern Hemisphere (Table 1), yet none of these reports have been confirmed using DNA sequence data. The fungus has been classified as an A1 quarantine pathogen (Code: CERSPD) by the European and Mediterranean Plant Protection Organization (EPPO) for regions such as Argentina, Brazil, Chile, and Uruguay (EPPO, 2002). Although there have been reports of *P. pini-densiflorae* in Brazil, Australia and New Zealand (EPPO, 2002), the pathogen did not persist in these areas or the reports were shown to be invalid (Minott, 2018).

Importation of infected *Pinus* planting stock has most likely been the route of introduction for *P. pini-densiflorae* into Africa and Asia (Sullivan, 2010). In Australia, a threat analysis based on the level of trade with different countries predicted that *P. pini-densiflorae* has a 53% likelihood of becoming a threat to Australian plantations (Paini, 2011). Assessments such as these will be useful in refining importation procedures and identifying high risk routes that may require intense inspection before plant material enters a country.

4.1.4 Host range

Pseudocercospora pini-densiflorae was described causing death in nurseries of *P. pinaster, P. thunbergii, Pinus luchensis* Mayr and its eponym, *P. densiflora* (Ito, 1972). It has mainly been reported infecting late nursery stock and small trees, causing severe defoliation that may often lead to seedling death (Gibson, 1979). Trials using seedlings of varying ages showed that susceptibility depended on the age of the seedling and host species (Ito, 1972).

Initial reports of *P. pini-densiflorae* in the Southern Hemisphere were mainly on *P. radiata* (Evans, 1984). Later, in South Africa, the pathogen was reported on *Pinus canariensis* C.Sm. D.C., *Pinus wallichiana* A. B. Jackson (previously *Pinus griffithii*), *P. pinaster* and *P. radiata* with ages ranging from 3 to 25 years (Ivory and Wingfield, 1986; Crous et al., 1990). In his report on foliage pathogens of *Pinus* spp. in the tropics, Ivory (1994) found that *P. pini-densiflorae* caused severe defoliation in older *P. radiata, Pinus roxburghii* Sarg. and *P. canariensis* trees. Other hosts reported from the Southern Hemisphere include *P. oocarpa, P. kesiya, P. elliottii* and *P. halepensis* Mill. (Ivory, 1994). The presence of the pathogen in subtropical habitats and its ability to thrive in less humid and warmer conditions on a variety of *Pinus* hosts suggests that it remains a threat to non-native plantations in regions of the Southern Hemisphere (Evans, 1984).

4.1.5 Biology and symptoms

Brown needle disease infection commences on the lower branches and progresses upwards into the crown (Fig. 1A) (Ivory and Wingfield, 1986). Conidia germinate in saturated water conditions and survive for approximately 48 hours (Gibson, 1979). The mechanism by which the fungus enters the plant is unknown but once it has invaded the tissue, it remains within the needle as mycelium during unfavourable conditions (Gibson, 1979). Latency can last between 13 to 60 days, depending on the density of inoculum and the environmental conditions (Suto, 1984).

In early infections, a small green lesion or band may be seen on the needle surface. These bands turn yellowish over time and eventually become grey-brown (Ito, 1972; Ivory and Wingfield, 1986). Blighted needles become shrunken and dry and may remain on the tree unless disturbed by intense winds and rain (Fig. 1B) (Ivory and Wingfield, 1986). Eventually, several black conidiomata develop that give a sooty, black appearance to the needles (Fig. 1C) (Ito, 1972). During warm and damp

weather, olivaceous clumps of conidia develop which are spread to surrounding trees by rain splash (Ivory and Wingfield, 1986).

Morphological variation exists between isolates of *P. pini-densiflorae* collected from *Pinus* spp. and several spore forms have been reported (Fig. 1D-E) (Ivory and Wingfield, 1986; Ivory, 1994). This was evident in the differences observed between isolates of *P. pini-densiflorae* collected from Africa, Jamaica and Asia, which Evans (1984) attributed to the variation in environmental conditions or host interactions in these countries. It will be important to determine whether these represent different ecotypes of *P. pini-densiflorae* or cryptic taxa.

4.1.6 Future research

Phylogenetic studies of the genus *Pseudocercospora* showed that *P. pini-densiflorae* falls within the clade *Pseudocercospora sensu stricto*. However, the genus still requires taxonomic revision (Crous et al., 2013). Due to the importance of this species to commercial plantations of non-native *Pinus* spp. in subtropical and tropical regions, it is suggested that recollection, culturing, and sequencing of the fungus using the ITS, translation elongation factor 1α (*TEF*1) and actin (*ACT*) regions should be undertaken to ensure accurate identification of this pathogen (Quaedvlieg et al., 2012).

4.2. Dothistroma needle blight

4.2.1 Introduction

Dothistroma needle blight (DNB), commonly also referred to as red band needle blight, first emerged as a serious problem in the 1950s to 1960s on non-native *P. radiata* plantations in the Southern Hemisphere (Gibson, 1972; Ivory, 1987). Since then, the disease has been recorded from all continents, except Antarctica (Bradshaw, 2004; Drenkhan et al., 2016; Barnes et al., 2022). Dothistroma needle blight is caused by either one of two ascomycete fungi: *Dothistroma septosporum* and *D. pini* (Barnes et al., 2004; Barnes et al., 2016). Only *D. septosporum* has been reported from the Southern Hemisphere, where DNB has had a major impact on commercial plantations of non-native *Pinus* spp.(Barnes et al., 2022).

4.2.2 Taxonomy

Historically, the causal agent of DNB has undergone multiple taxonomic changes resulting in numerous interchangeable names that have been accurately summarised by Barnes et al. (2016). Discrepancies in morphology led to a phylogenetic analyses that clarified that DNB is caused by two distinct species: *D.*

septosporum and *D. pini* (Barnes et al., 2004). These species produce indistinguishable symptoms and may even be isolated from the same needle (Barnes et al., 2011). The only means to accurately distinguish between the two species are by DNA-based molecular methods such as DNA comparisons of different gene regions, conventional and quantitative PCR, and ITS-RFLPs (Pehl and Wulf, 2001; Barnes et al., 2004; Ioos et al., 2010; Schneider et al., 2019).

4.2.3 Distribution in the Southern Hemisphere

Dothistroma pini has been reported in 17 countries across Europe and USA and seems confined to the Northern Hemisphere, however, evidence suggests that its geographical range may be expanding (Drenkhan et al., 2016; Mullett et al., 2018). *Dothistroma septosporum* (Code: SCIRPI), on the other hand, has been reported in 48 countries from the northern and Southern Hemispheres (Barnes et al., 2022). In the Southern Hemisphere it has been reported from Africa, Oceania and South America (Table 1). In Africa, DNB was first reported in Rhodesia (now Zimbabwe) in the 1940s and has since spread to seven other countries, but the causal agent of these outbreaks has only been confirmed using molecular techniques from Kenya and South Africa (Barnes et al., 2004). *Dothistroma septosporum* has been reported from eight countries in South America, for which only half have been confirmed using molecular techniques (Table 1). In New Zealand, Dothistroma needle blight was first discovered in 1964 and had spread throughout the island by 1967 (Gilmour, 1965; Drenkhan et al., 2016). Since then, *D. septosporum* has been confirmed in New Zealand and Australia (Barnes et al., 2004), while its presence in Papua New Guinea still requires confirmation (EPPO, 2015).

A recent worldwide study using microsatellite markers to determine the population history of *D. septosporum* revealed that majority of the introductions into the Southern Hemisphere were from population clusters in Western Europe. The exception is for South Africa, where multiple introductions of *D. septosporum* have occurred from several regions across Europe (Mullett et al., 2021). Introduction of these pathogen lineages bear a resemblance to the importation and establishment of *Pinus* plantations, especially the widely distributed *P. radiata* (Barnes et al., 2014; Mullett et al., 2021).

Dothistroma spp. are heterothallic and sexual recombination by outcrossing may result in new haplotypes that are able to overcome host resistance and adapt to new environments (Groenewald et al., 2007; Giraud et al., 2008). The sexual state of

D. septosporum has been reported from three countries in Africa; Kenya, Tanzania and Zimbabwe (Drenkhan et al., 2016). The presence of both mating types has, however, been confirmed only in South Africa and Kenya (Barnes et al., 2014). This is consistent with the moderate levels of genetic diversity seen in these populations, contrasting with regions such as New Zealand where only a single mating type is present and the population has remained clonal for approximately 60 years (Groenewald et al., 2007; Barnes et al., 2014; Drenkhan et al., 2016). In South America, population genetic studies have revealed the presence of both mating types from Colombia (Barnes et al., unpublished). In contrast, only a single type was confirmed in Ecuador and Chile (Mullett et al., 2021).

4.2.4 Host range

A detailed account of the host range for both *Dothistroma* spp. has been summarised by Drenkhan et al. (2016). The disease (DNB) has been reported on 113 host taxa, the majority of which belong to *Pinus* (Barnes et al., 2022). *Dothistroma septosporum* can infect 56 taxa across six genera. In contrast, D. *pini* has been reported only on 19 *Pinus* spp. and one species of *Picea* (Drenkhan et al., 2016; Ondrušková et al., 2017; Jánošíková - Hečková et al., 2018; Matsaikh et al., 2018). For some cases of DNB, the causal agent has not been verified using molecular techniques, meaning that the host range for these pathogens may be wider than is currently reported (Barnes et al., 2022).

In the Southern Hemisphere, DNB has severely impacted plantations of *P. radiata* and in many regions of Africa, this species needed to be substituted with *P. patula* (Gibson, 1972). Similarly, in New Zealand, *P. nigra* and *P. ponderosa* were abandoned for commercial use and DNB on *P. radiata* is controlled by aerial fungicide sprays and resistance breeding (Bulman et al., 2013). Species of *Pinus* have shown varying levels of susceptibility to *D. septosporum* infection (Ades and Simpson, 1991; Drenkhan et al., 2016; Mesanza et al., 2021). This is especially true for *Pinus* spp. native to Mesoamerica. Studies in Colombia, relating to DNB outbreaks, found that *P. maximinoi* and *P. patula* are the most tolerant to infection by the pathogen, whereas susceptibility in *P. tecunumanii* showed intraspecific variation depending on the provenance of the trees (Rodas et al., 2016).

Variability in disease tolerance has also been reported for the same *Pinus* spp. in different countries (Drenkhan et al., 2016). For example, it was found that *P. oocarpa* in Colombia is highly susceptible to *D. septosporum* infection, whereas in Kenya this

species was classified as only moderately susceptible (Ivory, 1968). This is important given the increased interest in Mesoamerican *Pinus* spp. for commercial forestry in the Southern Hemisphere. Various explanations exist for the inconsistency observed in disease severity, but variation in the environmental or pathogenic strain seem to be the most plausible (Drenkhan et al., 2016).

4.2.5 Biology and symptoms

Dothistroma needle blight is seriously affected by climatic conditions, as it flourishes in mild temperatures, high light intensity and most importantly, high levels of precipitation (Bradshaw, 2004). In regions such as Chile, East Africa and New Zealand, rainfall is a major predictor of the development of DNB (Gibson, 1972). However, in areas with a warmer climate, multiple generations of the pathogen can occur in one growing season, thus increasing the epidemic potential of these pathogens (Bulman et al., 2013).

Dothistroma spp. infect needles of all ages, generally starting at the bottom of the tree and moving up into the crown (Fig. 2A) (Bulman et al., 2013). Conidia (Fig. 2E) are dispersed mainly to adjacent trees by water splash and germinate on the needle surface, producing germ tubes that enter the needle via the stomata (Gadgil, 1967; Bradshaw, 2004). Hyphae proliferate in the mesophyll cells and surrounding intercellular spaces (Bradshaw, 2004; Kabir et al., 2015a, b; Fraser et al., 2016).

A yellow to water-soaked lesion can be observed at the point of infection (Fig. 2B). This is a result of the pathogen inducing necrosis of the surrounding tissues as it invades and by the secretion of the mycotoxin, dothistromin (Bradshaw, 2004; Kabir et al., 2015b; Drenkhan et al., 2016). During humid conditions, the stromata develops and ruptures the host cuticle, resulting in a flap of epidermal tissue over an erumpent, black fruiting body (Fig. 2C) (Bradshaw, 2004). Often a brick-red band can be seen at the fruiting body (Fig. 2C) due to the accumulation of the dothistromin (Fig. D) (Shain and Franich, 1981; Bradshaw, 2004; Kabir et al., 2015b). Needles begin to die from the site of infection to the distal region and are shed prematurely (Bulman et al., 2013).

The sexual cycle of *D. septosporum* occurs mainly on necrotic needles attached to the tree or that were cast. Ascospores are dispersed to new hosts primarily by wind (Evans, 1984; Dale et al., 2011; Mullett et al., 2017). Once the ascospores land on the needle surface, they germinate from both ends and the infection is initiated (Karadžić, 1989b). Small, dark, globose structures can be observed rupturing the epidermic at

advances stages of infection and the pathogen overwinters in attached or cast needles (Barnes et al., 2022).

4.2.6 Future research

Dothistroma needle blight is one of the most important and widespread diseases of *Pinus* spp. It is, therefore, not surprising that it is being extensively researched to alleviate its impact on commercial plantations in the northern and Southern Hemispheres. Molecular techniques have enhanced our understanding of the causal agents, *D. septosporum* and *D. pini*, and while these pathogens are morphologically indistinguishable, they are genetically distinct, and their distribution and host range varies considerably.

An intriguing question that remains a knowledge gap is why *D. pini* has never been introduced into the Southern Hemisphere. This is despite the fact that *D. pini* has been documented in the same areas as *D. septosporum* in the Northern Hemisphere in the past (Queloz et al., 2014; Ortíz de Urbina et al., 2017; Matsiakh et al., 2018). The geographic and host range of *D. pini* seems to be expanding and its potential to spread and establish in the Southern Hemisphere should be investigated.

The impact of biotic and abiotic factors on the development of DNB and how it might contribute to the inconsistencies seen in host susceptibility is still not well understood. Disease tolerance in species and provenances of *Pinus* spp. varies across different sites. This demonstrates the importance of understanding the interactions between the host, pathogen and environment and the outcome that these interactions have on disease severity. This information is important to develop durable control strategies against DNB.

Under future climate change scenarios, the suitable area of *Dothistroma* species in the Southern Hemisphere may decrease in non-commercial regions. However, no disease alleviation was observed in the regions presently growing *Pinus* spp. (Watt et al., 2011). Population genetic studies on *D. septosporum* have aided in the understanding of the evolutionary history, global and local population structures and possible migratory routes for this pathogen. These studies are noticeably lacking for *D. pini,* given the possibility of an accidental introduction of this pathogen into the Southern Hemisphere, this should be an important area of future research.

4.3 Brown spot needle blight

4.3.1 Introduction

Brown spot needle blight (BSNB), caused by the ascomycete *Lecanosticta acicola,* was first discovered by de Thümen in 1878 in the northern part of the United States of America (USA) on *Pinus echinata* (De Thümen, 1878). Longleaf pine (*Pinus palustris*), in particular, has been severely affected by this disease and may suffer from severe defoliation when infected at the grass stage of early growth (Siggers, 1934). This disease is widely distributed across Europe and North America. Recently, several other species of *Lecanosticta* have been described from *Pinus* spp. native to Mesoamerica (van der Nest et al., 2019b; Theron et al., 2022). Given the increasing popularity of these *Pinus* spp. for commercial forestry in the Southern Hemisphere, there is a serious risk of one or more of these pathogens being introduced together with germplasm in the future.

4.3.2 Taxonomy

Lecanosticta acicola is the type species of the genus that was erected by Sydow and Petrak (1922). However, the species name has undergone numerous revisions that have been summarised in a review by van der Nest et al. (2019a). Eight other species have been described that belong to this genus, seven of which seem to be confined to Mesoamerica (Evans, 1984; Marmolejo, 2000; Quaedvlieg et al., 2012; van der Nest et al., 2019b). The impact these other species of *Lecanosticta* will have on commercial forestry, if introduced into new areas, is unknown.

4.3.3 Distribution

Lecanosticta acicola is widespread in the Northern Hemisphere across 31 countries from the USA, Canada, parts of Europe, Asia and Central America (van der Nest et al., 2019a; Raitelaitytė et al., 2020). Based on sequences of the *TEF*1 gene region, there are three distinct lineages. Canada, northern regions of USA, Central and Northern Europe form part of one lineage. A second lineage is found in China, Colombia, France, Japan, Spain, South Korea and southern regions of the USA (Janoušek et al., 2016). Isolates from this lineage were responsible for a severe outbreak of BSNB in non-native *Pinus* plantations in Colombia in the past (Evans, 1984). A third lineage of *L. acicola* seems to be confined to Mexico (van der Nest et al., 2019b).

Lecanosticta acicola has been reported from plantations of non-native *Pinus* spp. in South Africa and Colombia. However, the report from South Africa could not

be verified and the pathogen is considered absent from that country (EPPO, 2021). The initial report in Colombia was by Gibson (1980) and this was followed-up by Evans (1984) when the sexual and asexual state of the pathogen was identified based on their morphological characteristics. The only molecular evidence confirming the presence of *L. acicola* in Colombia was by Janoušek et al. (2016) when part of the *TEF*1 gene region was sequenced for a small number of isolates from *P. caribaea*. Due to the severity of BSNB in the Northern Hemisphere, the pathogen is considered a significant quarantine risk in North Africa and has been rated an A1 quarantine status (Code: SCIRAC) in Argentina, Bahrain, Brazil, Chile and Uruguay in the Southern Hemisphere (EPPO, 2021).

Since 2019, disease symptoms resembling those of BSNB have been reported in young *P. maximinoi* (Fig. 3A) plantations in Colombia and more recently severe defoliation of *P. patula* and *P. tecunumanii* has also been reported (Rodas and Wingfield, 2020). With the recent characterisation of new species of *Lecanosticta* on Mesoamerican *Pinus* spp., the causal agent of this outbreak was investigated and confirmed as *Lecanosticta pharomachri* van der Nest, M.J. Wingf.& I. Barnes (Theron et al., 2022) (See Chapter 2). This is the first report of the species outside Mesoamerica (Guatemala and Honduras) and of the sexual state of this species which was formally described in the study by Theron et al. (2022). The high level of genetic diversity seen in the *TEF*1 gene region sequences for the *L. pharomachri* isolates from Colombia suggests that multiple introductions of this species may have occurred, along with the Mesoamerican host material, in the past (Theron et al., 2022).

4.3.4 Host range

Lecanosticta acicola has been reported on 54 different *Pinus* hosts from native and non-native regions (van der Nest et al., 2019a; Mesanza et al., 2021). In Colombia, the pathogen was first identified on *P. radiata, P. elliottii* and *P. patula* and later confirmed on *P. caribaea* (Gibson, 1980; Evans, 1984; Janoušek et al., 2016). *Lecanosticta pharomachri* was initially described on *P. tecunumanii* and *P. oocarpa* (van der Nest et al., 2019b) but in Colombia this species was reported for the first time on *P. patula* and *P. maximinoi* (Theron et al., 2022). Although *L. acicola* has been reported on these same four hosts in Mesoamerica, these reports have not been confirmed using molecular data and may represent other species of *Lecanosticta* (van der Nest et al., 2019b; Theron et al., 2022).

Due to the absence of both *L. acicola* and *L. pharomachri* in all other regions of the Southern Hemisphere, little is known regarding the impact they may have on these plantations. However, it has been suggested that in tropical and subtropical conditions, where susceptible hosts are planted mainly in monoculture, an introduction of these pathogens could be detrimental (Evans, 1984; van der Nest et al., 2019a).

4.3.5 Biology and symptoms

Lecanosticta acicola can be found in either the sexual or asexual stage. Asexual conidia are released during high humidity conditions when light and temperature are favourable. The conidia germinate and penetrate through the stomata of mainly lower canopy needles (Fig. 3A), producing light grey-green, irregular spots at the site of infection (Hedgcock, 1929; Kais, 1975). Over time these spots turn light brown with yellow margins and, depending on the host, may appear resin soaked (Fig. 3B) (Wolf and Barbour, 1941; Skilling and Nicholls, 1974). The disease caused by *L. acicola* is aptly named BSNB due to these brown spots observed on the needle surface.

The mycotoxins, LA-I and LA-II, produced by *L. acicola* (Fig. 3C) can be observed in cultures of the fungus and has found to result in a sensitivity response in highly susceptible species of pine (Ye and Qi, 1999). Tissue studies have revealed that these toxins play a role in the destruction of mesophyll cells at the site of infection (Jewell, 1983; Jianren and Gaofu, 1999). This most likely aids the pathogen in colonizing the surrounding tissue during infection.

As the infection progresses, needles die from the tip to the base and are eventually cast (Skilling and Nicholls, 1974). The fungus proliferates in the mesophyll cells of the needle tissue and produces conidiophores bearing light brown conidia (Fig. 3D) towards the leaf surface. These fruiting bodies exert pressure on the epidermis, causing it to rupture in parallel slits enclosed by an epidermal flap (Jewell, 1983). During wet weather, conidia are released and disseminated short distances by rain splash (Wolf and Barbour, 1941).

In the sexual cycle, ascostromata containing asci develop at the tips of living or dead needles. Ascospores are released from the asci and are disseminated over long distances by wind and rain, often triggering major epidemics (Siggers, 1944; Henry, 1954; Jewell, 1983). The development of asci and ascospores, although rare, has been reported in several countries, including Colombia (Evans, 1984).

Depending on the strain of *L. acicola*, the asymptomatic phase can last from several days to three months and could result in the accidental introduction of the pathogen with infected plant material (Skilling and Nicholls, 1974; van der Nest et al., 2019a). Symptoms of BSNB also bear similarities to DNB caused by *D. septosporum* and *D. pini.* Given the global distribution of these pathogen in the northern and Southern Hemisphere, it is important to accurately discern between them and *L. acicola* (Barnes et al., 2016).

4.3.6 Future research

Lecanosticta acicola is the only species known to cause the disease BSNB. However, the recent outbreak of disease caused by *L. pharomachri* in Colombia and the discovery of additional cryptic species from Mesoamerica, suggest that previous reports of *L. acicola* should be revised. The *TEF*1 gene region, used in combination with ITS, has been identified as useful barcoding loci to distinguish between the different species of *Lecanosticta* (Quaedvlieg et al., 2012; van der Nest et al., 2019b; Theron et al., 2022).

The report of *L. pharomachri* on non-native *Pinus* spp. in Colombia is the first for this pathogen outside its apparent native range. Variability observed in the sequence data for the isolates from Colombia suggests that multiple introductions of this pathogen may have occurred into that country in the past (Theron et al., 2022). Population genetics studies for *L. pharomachri* will aid in elucidating the genetic diversity and structure of this population, mode of reproduction and pathways of introduction into Colombia.

These species could pose a potential risk to commercial plantations of non-native *Pinus* spp., especially on the Mesoamerican species now being widely introduced into the Southern Hemisphere for commercial forestry. This emphasises the need for caution when importing planting material for future plantation purposes (van der Nest et al., 2019a). An available LAMP assay may aid in the rapid and accurate identification of these pathogens for routine phytosanitary control (Aglietti et al., 2021).

4.4 Cyclaneusma needle cast

4.4.1 Introduction

Cyclaneusma needle cast, caused by *Cyclaneusma minus* (Butin) Di Cosmo, Peredo & Minter (previously *Naemacyclus minor* Butin) and *C. niveum* Minter

(previously *Naemacyclus niveus* [Persoon] Saccardo), is found on various *Pinus* spp. around the world. These ascomycete fungi have had major repercussions on the Christmas tree industry in Eastern Pennsylvania, USA eliminating the commercial use of *P. sylvestris* in this region (Wenner and Merrill, 1986). In New Zealand and Australia, outbreaks caused by *Cyclaneusma minus* negatively impacted commercial plantations of *P. radiata* (Fig. 4A) (van der Pas et al., 1984; Choi and Simpson, 1991). In New Zealand, the loss attributed to Cyclaneusma needle cast on *P. radiata* was estimated at NZD$38 million per annum (Bulman, 2009).

4.4.2 Taxonomy

Initially, the needle cast outbreaks were attributed only to *N. niveus,* but based on morphology and host-relationships, a distinct separation could be made between this species and *N. minor* (Butin, 1973). Nomenclatural revision of the genus, *Naemacyclus* Fuck., was undertaken by DiCosmo et al. (1983) after it was found that these species were not conspecific with the type species, *Naemacyclus pinastri* (Lacroix) Fuck. Therefore, the generic name, *Cyclaneusma* DiCosmo, Peredo & Minter*,* was erected to accommodate the two needle cast fungi.

4.4.3 Distribution in the Southern Hemisphere

Cyclaneusma minus is considered the more economically important pathogen of the two species and has been recorded in 39 different countries (CABI, 2022), 11 of which are in the Southern Hemisphere (Table 1). Millar and Minter (1980) provided descriptions of the disease, host report and geographical distribution of *C. minus* based on morphological characteristics. Many of these reports are still based on the original morphological descriptions and the identity of the pathogen has been verified using molecular techniques only in Kenya, Australia, New Zealand and Chile (Prihatini et al., 2014; Vu et al., 2019).

There is uncertainty regarding the geographical distribution of *C. niveum* since the taxonomic revisions were implemented. Discrepancies exist in regions where *C. niveum* was reported predating this revision. For example, both *C. niveum* and *C. minus* have been reported in Kenya on *P. radiata* and *P. patula* (Nattrass, 1961; Gibson, 1970; Marmolejo and Minter, 2006). However, in his delineation, Butin (1973) noted that these *Pinus* spp. are outside the host range of *C. niveum.* Accordingly, records of *C. niveum* occurring on *P. radiata* in New Zealand were investigated and later attributed to *C. minus* (Gadgil, 1984). *Cyclaneusma niveum* has been recorded

in five countries from the Southern Hemisphere, yet no molecular verification has been performed for this species (Table 1).

4.4.4 Host range

Cyclaneusma needle cast has been recorded on 20 different *Pinus* spp. (CABI, 2022). Butin (1973) initially separated the two species by host range, however, the host lists for the two species has since shown not to be mutually exclusive (Millar and Minter, 1980; Minter and Millar, 1980a). In the Southern Hemisphere, disease outbreaks mainly occur on *P. radiata* and the most severe outbreaks were reported from Australia and New Zealand (Gadgil, 1984; Bulman, 1988; Choi and Simpson, 1991; Bulman, 2009).

In Argentina, a disease locally referred to as 'red band' was reported on *P. contorta, P. jeffreyi, P. ponderosa* and *P. radiata*. The causal agent was diagnosed, based on the symptoms on the surface of the needles, as *C. minus* and not *Dothistroma* (Rajchenberg et al., 1995). It is important to take note that these two species have been reported to co-occur and to date the presence of neither species has been confirmed in Argentina (Podger and Wardlaw, 1990; Choi and Simpson, 1991). Therefore, it is important to monitor and confirm the presence of such fungi using molecular techniques for targeted control strategies.

4.4.5 Biology and symptoms

Symptoms of Cyclaneusma needle cast can occur on trees of varying age and size and mainly under conditions of high humidity and mild temperatures (Wenner and Merrill, 1986). Needle cast occurs during spring and autumn in the first year of canopy closure (Gadgil, 1984; Karadžić, 1989a; Podger and Wardlaw, 1990). At the site of infection of the stomata, small, yellow spots begin to develop as the fungus infiltrates the parenchymatous cells, destroying the chloroplast and distorting the epidermis (Podger and Wardlaw, 1990). The spots later coalesce, and chlorosis progresses from the site of infection to the distal and basal ends of the needle. Distinct, transverse, reddish-brown bands may be seen and at this stage the needles readily detach and are cast prematurely in summer and autumn (Gadgil, 1984).

Needle cast caused by *Cyclaneusma* spp. affects needles at one-year or older in the central and lower regions of trees (Fig. 4A). Once needles are shed, the crowns of the trees are thin and tufted, with only newly flushed foliage being present (Gadgil, 1984; Podger and Wardlaw, 1990). Dead needles bear numerous apothecia that are a waxy, tan colour when young but eventually mature and rupture the needle surface,

producing “hinges” along the sides that resemble the colour of the needle surface (Gadgil, 1984). After rainfall or periods of high humidity, the “hinges” are pushed back to expose an arched layer of straw-coloured ascospores (Fig. 4B).

Ascospores (Fig. 4D) are only liberated from the apothecia during periods of rainfall, but has been found to occur throughout the year, mainly on dead and detached needles (Gadgil, 1984; Wenner and Merrill, 1986). Variation has been seen in the latency period for symptom development and spore dispersal, and needles that appear asymptomatic have been shown to contain a high level of infective propagules (Prihatini et al., 2016; Behnke-Borowczyk et al., 2019). Situations such as these may contribute to the involuntary dispersal of infectious plant material.

4.4.6 Future research

Investigations into the variability of the populations of *Cyclaneusma* spp. in New Zealand revealed two morphotypes of *C. minus* on *P. radiata*. *Cyclaneusma minus* ‘verum’ shared attributes with *C. minus* (Fig. 4C) as described by Butin (1973), whereas, *C. minus* ‘simile’ shared cultural characteristics with *C. niveum* (Dick et al., 2001). Subsequently, similar morphotypes have also been found in Australia and a call for further research led to a multigene phylogenetic analysis of these isolates. The results of the study confirmed the morphological observations and the morphotype, ‘simile’, is now considered a new species which is yet to be formally described (Prihatini et al., 2014).

Cyclaneusma species have been considered to reside in the *Rhytismataceae* in the *Rhytismatales* (Gernandt et al., 2001; Schoch et al., 2009). However, it has been suggested they are more correctly members of the order *Helotiales incertae sedis* (Lantz et al., 2011). Substantial variation in the severity of Cyclaneusma needle cast suggests the need for a global investigation into the taxonomy, distribution, pathogenicity and biology of the two, or possibly three, *Cyclaneusma* species.

4.5 Lophodermium needle cast

4.5.1 Introduction

Lophodermium spp. are ascomycetous fungi that have been isolated from a variety of hosts, but most studies have focused on species infecting *Pinus* spp. due to their conspicuous symptoms on needles. Approximately 38 species of *Lophodermium* have been described from pines and many more are being discovered and described (Ortiz-García et al., 2003; Tanney and Seifert, 2017; Salas-Lizana and Oono, 2018b).

Lophodermium seditiosum is the only major pathogen in the genus, causing needle cast of *Pinus* spp. in nurseries and plantations across North America and Europe (Skilling and Nicholls, 1975; Minter et al., 1978; Lazarev, 1984; Kowalski, 1989). In 1966, an outbreak of Lophodermium needle cast arose throughout North America after infected nursery stock of *P. sylvestris* and *P. resinosa* Ait. was distributed throughout the country (Skilling and Nicholls, 1975).

4.5.2 Taxonomy

Lophodermium needle cast disease was reported for the first time in 1799 and was attributed to *Hysterium pinastri* Schrad. The genus, *Lophodermium*, was later erected by Chevallier to accommodate this pathogen and many other morphologically similar species (Chevallier, 1822). Over the years, many species have been described in this genus and the documented distribution of *Lophodermium* spp. increased worldwide (Minter, 1981).

Between the 1960s and 1970s, researchers began to discover discrepancies in the age of the needles being infected and culture characteristics of *L. pinastri* (Millar and Watson, 1971; Skilling and Nicholls, 1975; Minter, 1981). Minter et al. (1978) performed isolations from needles collected across North America, Europe and Australia and they isolated three species (*L. pinastri, L. conigenum* and *L. pini-excelsae*) previously known and a novel species, *L. seditiosum* Minter, Staley & Millar. It was later shown that *L. seditiosum* is pathogenic to *P. sylvestris* seedlings and trees and this species was named as the causal agent of the long-known Lophodermium needle cast (Minter and Millar, 1980b).

4.5.3 Distribution in the Southern Hemisphere

Lophodermium seditiosum has only been reported from Chile and South Africa in the Southern Hemisphere, but these reports have relied only on morphological features (Rack, 1980; Roux and Lundquist, 1984). Many endophytic *Lophodermium* spp. have been reported in the Southern Hemisphere (Table 1), often inhabiting needles that were killed prematurely by another pathogen or due to the effects of abiotic factors (Gibson, 1972; Minter and Millar, 1980b; Diwani and Millar, 1990; Choi and Simpson, 1991; Johnston and Park, 2007). One of the earliest reports of such endophytic *Lophodermium* infections in the Southern Hemisphere is that of *L. pinastri* reported between 1893 and 1900 in South Africa (Zahn and Neethling, 1929). Molecular confirmation of these endophytic *Lophodermium* spp. have only been

performed in Australia and New Zealand in the Southern Hemisphere (Johnston et al., 2003; Ortiz-García et al., 2003; Prihatini et al., 2015).

Lophodermium pinastri was reported amongst a complex of endophytic species responsible for outbreaks of Spring needle cast (SNC) in 6 to 7 year old *P. radiata* plantations in Tasmania (Prihatini et al., 2015). Spring needle cast has not been reported in New Zealand, even though the presence of *L. pinastri* has been confirmed on *P. radiata.* In addition, isolates of *L. pinastri* from New Zealand and Europe seem to be genetically distinct from the isolates collected in Australia (Johnston et al., 2003; Prihatini et al., 2016). This is an agreement with previous work by Reignoux et al. (2014), suggesting that several cryptic taxa are present within the morphological species treated as *L. pinastri.*

Minter (1981) suggested that the two species, *L. australe* and *L. conigenum* could be cryptic. This has since been confirmed by several phylogenetic analyses that have shown these species to be conspecific (Johnston et al., 2003; Salas-Lizana and Oono, 2018b, a). Exhaustive sampling of these species from plantations or forests of native *Pinus* spp. in the Northern Hemisphere and molecular analyses are required to determine how many *Lophodermium* species there are and what their role is in needle-cast disease of *Pinus* spp.

4.5.4 Host range

Lophodermium seditiosum mainly infects seedlings and results in a significant reduction in height, tree diameter and vigour and, in severe cases, death (Fig. 5A) (Ostry and Nicholls, 1989; Jansons et al., 2016). This pathogen has been reported on over 20 different *Pinus* spp., none of which are currently prominent in plantation forestry in the Southern Hemisphere (Ugalde and Perez, 2001; Lazarev et al., 2007; Farr and Rossman, 2022). The report of *L. seditiosum* in Chile was on known hosts of the pathogen, including; *P. cembra, P. contorta, P. nigra* and *P. sylvestris* (Minter, 1981; Butin and Peredo, 1986). In South Africa, the pathogen was reported on *P. canariensis* and *P. oocarpa*, two species that are not known hosts of *L. seditiosum,* and for which its presence requires molecular confirmation (Minter, 1981).

4.5.5 Biology and symptoms

Ascospores of *L. seditiosum* are released in late summer and colonize primary and secondary needles mainly on the lower branches of trees in humid to wet conditions (Fig. 5A) (Sinclair and Lyon, 2005). After the spores germinate, the fungus penetrates the needles using the turgor pressure of a melanised appressoria and then

incubates inside the needle for five to six months (Minter and Millar, 1980b; Karadžić, 1989a). As the fungus infiltrates the epidermal and hypodermal cells, yellow-brown spots develop at the site of infection. Over time the spots turn brownish red, and needles are killed and cast (Gibson, 1979; Diwani and Millar, 1984).

The pathogen overwinters in dead needles and during wet, humid conditions of spring and summer, elongated, shiny black ascocarps develop. These ascocarps have a characteristic slit down the centre and are occasionally flanked by diffuse, brown stromatic lines (Fig. 5B, C) (Minter et al., 1978). The asexual stage has been identified as *Leptostroma* Fr. ex Fr., but thus far its role is unknown (Minter, 1980).

4.5.6 Future research

A bias in taxon sampling is evident for regions of the Northern Hemisphere, resulting in a gap in the knowledge on what impact *Lophodermium* spp. may have on plantations of non-native *Pinus* spp. However, before studying these populations it is important to ensure that they do not include cryptic taxa. Ascocarp development is the main morphological feature used to distinguish between different species of *Lophodermium* (Minter, 1981). Given the cryptic nature now observed amongst species residing in *Lophodermium*, ascocarp development as an identification tool may not be useful to differentiate between the species. Development of the ascocarp is not yet well understood and neither is there much knowledge regarding the impact that different host anatomies may have on ascocarp development (Ortiz-García et al., 2003).

Due to the quarantine importance of *L. seditiosum* in New Zealand, a PCR-RFLP method was developed that can be used as a tool to differentiate endophytic *Lophodermium* species from the pathogen, *L. seditiosum* (Johnston et al., 2003). Studies have shown that *L. seditiosum* can be isolated from seed, but the mechanism with which the fungus enters the seed or whether it is still viable when the seed germinates is not yet well understood (Bentele et al., 2018). These questions and the involvement of endophytic species in needle-cast disease can only be answered once a fully resolved, well supported phylogeny has been constructed.

4.6 Diplodia shoot and tip blight

4.6.1 Introduction

Diplodia shoot and tip blight is a disease responsible for significant losses in nurseries and plantations of *Pinus* in the past (Swart and Wingfield, 1991). The causal

agent, an ascomycete named *Diplodia sapinea,* has been reported from many countries of the world, from natural and cultivated plantations in the Northern Hemisphere to commercial plantations of non-native *Pinus* spp. across the Southern Hemisphere (Gibson, 1979). A multigene phylogenetic analysis revealed that a morphotype of *D. sapinea* represents a discrete species, described as *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf (de Wet et al., 2003). Subsequently, this species has been reported from South Africa in the Southern Hemisphere and suggests that previous reports of *D. sapinea* need to be verified using DNA sequence data (Bihon et al., 2011b).

4.6.2 Taxonomy

The causal agent of Diplodia shoot and tip blight on *Pinus* spp., *Sphaeria pinea* Desm., was first described in 1842 in France and has since acquired many additional synonyms (Sutton and Dyko, 1989). The synonym *Diplodia pinea* (Desm.) J. Kickx f., established in 1867, became commonly used but was later replaced by *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. However, upon revision of the taxonomy it was found that *Sphaeropsis sapinea* and the type, *Sphaeria pinea,* are two distinct species and that the name *Diplodia sapinea* should be used for the pathogen (Phillips et al., 2013).

Four morphotypes of *D. sapinea* have been characterised (A, B, C and I) based on spore and culture morphology and later molecular techniques (Palmer et al., 1987; Burgess et al., 2001a; de Wet et al., 2002). Subsequently, multigene phylogenetic studies have shown that the I and B morphotypes represent discrete taxa, *Botryosphaeria obtusa* (Schwein.) Shoemaker and *D. scrobiculata,* respectively (Palmer et al., 1987; Burgess et al., 2001a; de Wet et al., 2003).

4.6.3 Distribution in the Southern Hemisphere

The earliest report of *D. sapinea* from the Southern Hemisphere is from South Africa in 1909 when the fungus was reported in plantations in the Cape region (Bancroft, 1911). Since then, the pathogen has been reported in 20 other countries across the Southern Hemisphere (Table 1). In Africa, the pathogen has been reported in 12 countries, for which seven have been verified using molecular methods (Stanosz et al., 1999; de Wet et al., 2000; Bihon et al., 2012a). From the Asian continent, the virulent C morphotype of *D. sapinea* was collected and described from Indonesia (de Wet et al., 2000; de Wet et al., 2002).

In New Zealand, Birch (1936) noted that *D. sapinea* was omnipresent in the forests but mainly resembled a saprophyte. In 1967 a large surge in disease incidence,

associated with mechanical- and frost damage, resulted in the outbreak of Diplodia tip blight in hundreds of hectares of *P. radiata* trees (Chou, 1976). Similarly, predisposing factors have also resulted in the increase in outbreaks in Australia (Marks and Minko, 1969). The presence of the pathogen has been verified using molecular techniques in both Australia and New Zealand (Stanosz et al., 1999).

The earliest report of the pathogen in South America was from Argentina in 1946 where it was found attacking buds, shoots and needles of non-native *Pinus* spp. (Saravi, 1950). In Brazil, the pathogen was also reported from São Paulo State in the 1940s after the introduction of *P. radiata* (Corrêa et al., 2011). Of the five countries in South America where *D. sapinea* has been reported, its presence has been verified, using DNA sequence data, in Argentina, Brazil, Chile and Uruguay (Bettucci et al., 2004; Wu et al., 2007; Corrêa et al., 2009; Bihon et al., 2012a).

Diplodia scrobiculata was thought to be confined to the Northern Hemisphere but has since been isolated from *P. patula* in South Africa (Burgess et al., 2004b; Bihon et al., 2011b). It is hypothesized that *D. scrobiculata* may have been introduced into South Africa with germplasm of *P. radiata* sourced from California (Bihon et al., 2011b). To date, this pathogen has only been reported from South Africa in the Southern Hemisphere.

A population study performed on isolates of *D. sapinea* from the northern and Southern Hemispheres found that the populations within each region were more related than between the two spheres, possibly due to the exchange of seeds or germplasm between the countries (Burgess et al., 2001b; Burgess and Wingfield, 2002; Slippers and Wingfied, 2007; Bihon et al., 2012a). Populations from Argentina and South Africa in particular are more diverse than the indigenous populations of the pathogen in North America and Indonesia (Smith et al., 2000; Burgess et al., 2001b; Bihon et al., 2012a). In Australia and New Zealand, the populations had an intermediate genetic diversity which could be explained by their strict quarantine regulations (Burgess et al., 2001b; Bihon et al., 2012a).

Diversity in the Southern Hemisphere populations may be explained by the occurrence of cryptic sexual recombination, even though the sexual state of the pathogen has not been found (Burgess et al., 2001b; Bihon et al., 2012a; Bihon et al., 2012b; Bihon et al., 2014). Evidence shows that *D. sapinea* did not originate in the native regions of its prominent host, *P. radiata,* and although it seems that the

countries in the Southern Hemisphere may share a common source of introduction, the origin is still unknown (Burgess et al., 2004a; Bihon et al., 2012a).

4.6.4 Host range

Diplodia sapinea has been reported on 50 different *Pinus* spp. and its distribution in the Southern Hemisphere closely mimics the introduction of its host (Palmer et al., 1987; Phillips et al., 2013; Decourcelle et al., 2015). The majority of the reports occur on *Pinus,* but recently the pathogen has also been reported on hosts belonging to other coniferous genera (Gibson, 1979; Sinclair and Lyon, 2005; Zlatković et al., 2017). The first report of the pathogen in South Africa was on *P. radiata* and *P. mugo* (Bancroft, 1911). The incidence of disease on *P. radiata* plantations then became prominent across many other regions in the Southern Hemisphere. In Swaziland (now Eswatini), a 600 ha compartment of *P. radiata* trees suffered a 50% loss due to infections (Wingfield and Knox-Davies, 1980) and in Australia such infection resulted in severe timber degradation in *P. radiata* plots (Marks and Minko, 1969). Intensive surveys need to be performed to determine the host range of these pathogens, especially for the Mesoamerican *Pinus* spp., of which *P. patula* has already shown to be highly susceptible (Rodas and Wingfield, 2020).

Differences in host susceptibility have been shown for areas with similar climates. In Eswatini, *P. patula* trees adjacent to infected *P. radiata* plantations seemed unaffected as compared to *P. elliotii* and *P. taeda* which showed signs of disease (Wingfield and Knox-Davies, 1980). Whereas, in South Africa, *P. patula* was found to be far more susceptible than both these hosts (Swart and Wingfield, 1991).

Although originally described as a non-pathogenic endophyte, it has been shown that *D. scrobiculata* can cause tip blight symptoms on several *Pinus* spp. (Bihon et al., 2011b; Hlaiem et al., 2019). Initial inoculation trials suggest that for a small number of isolates, the aggressiveness of *D. scrobiculata* matches that of *D. sapinea* (Bihon et al., 2011b; Manzanos et al., 2019). However, this is dependent on several factors and additional studies are required to better understand the differences in the relative virulence of the two species (Bihon et al., 2011b). Host species specificity has also been observed in *P. nigra* when infected with *D. sapinea* and *D. scrobiculata.* For the latter, the host initiated a defence response preventing the pathogen from colonizing the host tissue (Luchi et al., 2005). This suggests a form of pathogen recognition that may contribute to the limited distribution of *D. scrobiculata* in Europe and regions of the Southern Hemisphere (Slippers and Wingfied, 2007).

4.6.5 Biology and symptoms

Diplodia spp. are opportunistic pathogens that causes a range of different symptoms that are not only confined to the needles (Swart and Wingfield, 1991; Sinclair and Lyon, 2005). Tip blight, however, is the most common disease symptom that can affect young and mature trees (Fig. 6A) (Gibson, 1979). The pathogen overwinters in dead needles, stems, and pinecone scales until spring. In the spring, wet and warm weather permits the sporulation and dissemination of the conidia to adjacent trees by wind, rain-splash and insects (Swart and Wingfield, 1991; Sinclair and Lyon, 2005).

In wet periods of over 12 hours, the conidia of *D. sapinea* (Fig. 6F) germinate and infect needles, 1 to 3 years old, through the stomata (Li et al., 2019). Once inside the needle, the pathogen can remain latent for a few months while it proliferates in the substomatal tissue (Sinclair and Lyon, 2005). Symptoms become visible when a tree is exposed to stressful conditions, whether biotic or abiotic. In South Africa, a severe outbreak of die-back was seen across approximately 2000 ha of *P. patula* stands after a hailstorm with strong bouts of wind (Zwolinski et al., 1990). Wounds created by insect pests in New Zealand also aid in infection by creating an entry point for the fungus to easily access the succulent tissue of the trees. Other predisposing factors include pruning, water deficit, compacted soil, and frost damage (Gibson, 1979; Swart and Wingfield, 1991; Sinclair and Lyon, 2005; Li et al., 2019).

Needle growth becomes stunted as the fungus grows towards the base of the needle and invades the shoots. Once the fungus has invaded the shoots, the shoots dry out, become stunted and eventually die (Fig. 6B) (Gibson, 1979; Sinclair and Lyon, 2005). After approximately three weeks (in late summer) small, round, black pycnidia can be seen at the base of the needle, under the fascicle sheath, on the shoots and cones (Fig. 6C-D). These pycnidia may also be surrounded by grey-brown mycelium (Fig. 6E) and resin produced by the tree results in scattered clumps of dead needles in the canopy (Sinclair and Lyon, 2005).

Studies have shown that these pathogens are able to persist in asymptomatic trees and seeds (Smith et al., 2000; Flowers et al., 2001; Bihon et al., 2011a; Manzanos et al., 2017). Although some seed borne endophytes are present within its host from the time it germinates (Saikkonen et al., 2004; Shahzad et al., 2018), it is suggested that *D. sapinea* enters the *Pinus* seedlings in the nursery or after out-planting (Burgess et al., 2004b; Bihon et al., 2011c; Decourcelle et al., 2015; Larsson

et al., 2021). Nevertheless, asymptomatic infections may have important implications for the spread and emergence of disease outbreaks for these pathogens.

4.6.6 Future research

Diplodia sapinea is a well-known pathogen of *Pinus* spp. worldwide and it causes significant economic damage to stressed stands (Swart et al., 1987). However, since the discovery of *D. scrobiculata*, it has been found that *D. sapinea* and *D. scrobiculata* are able to coexist (Burgess et al., 2004b; Bihon et al., 2011b). Niche partitioning between these two species has not been well studied and, given that they are very similar in morphology, past reports of *D. sapinea* need to be confirmed using molecular techniques to ensure that the other species of *Diplodia* were not overlooked.

Even though the origin of these pathogens has not been confirmed, it is evident that the movement of plant material between Southern Hemisphere countries has contributed substantially to the genetic diversity of *D. sapinea.* Molecular tools have also confirmed that *D. sapinea* is able to reproduce sexually (Bihon et al., 2012a; Bihon et al., 2014). This is significant when considering its evolutionary potential to overcome management strategies and a concerted effort will need to be made to prevent the introduction of the opposite mating type or new genotypes into new areas.

Populations of *D. scrobiculata* from Mexico showed high genotypic diversity, which will need to be taken into consideration as Mesoamerican *Pinus* spp. are increasing in popularity in commercial forestry in the Southern Hemisphere. It is already known that, under certain environmental conditions, *P. patula* is susceptible to infection by *D. sapinea* (Gibson, 1979; Swart and Wingfield, 1991; Rodas and Wingfield, 2020). It will be important to determine the susceptibility of the other *Pinus* spp. to these pathogens before importing germplasm for future planting programmes.

4.7 Daño Foliar del Pino

4.7.1 Introduction and taxonomy

In the year 2004, unusual tree mortality was observed across 70 ha of six-year-old *P. radiata* stands in Chile. This increased drastically over the following two years and by the end of 2006, the disease had spread across 60, 000 ha. The disease, referred to as ‘Daño Foliar del Pino’ (DFP), caused the most severe disease outbreak Chile had ever seen (Fig. 7A) (Durán et al., 2008).

In 2007, *Phytophthora* selective media continuously yielded an unknown oomycete. This species was able to infect *P. radiata* in an inoculation trial and based

on DNA sequences (Durán et al., 2008) fell within Clade VI of the *Phytophthora* phylogeny constructed by Cooke et al. (2000). The novel species causing DFP was formally described in 2008 as *Phytophthora pinifolia* Alv. Durán, Gryzenh. & M.J. Wingf., the first known species of *Phytophthora* able to infect the needle and soft tissue of *Pinus* spp. (Durán et al., 2008)

4.7.2 Distribution in the Southern Hemisphere

Daño Foliar del Pino disease was first observed at the coastal areas of the Arauco province of Chile and has now spread across the country (Durán et al., 2010). Neither the disease or the pathogen have been recorded in any other native or non-native plantations around the world. One scenario for how the disease originated is that a host jump occurred from the native flora at the coastal region to the *P. radiata* trees in the proximity. However, these plantations have existed for over a century, making this phenomenon highly unlikely (Durán et al., 2008; Durán et al., 2010). Molecular studies have also found that the population in Chile is clonal, making a single introduction from an unknown area a more plausible explanation. The pathogen most likely originated in the native area of the host and the climatic conditions in Chile were optimal for the development of disease (Durán et al., 2010).

4.7.3 Host range

The disease severely affected *P. radiata* plantations along the coast of Chile but was not recorded on *P. pinaster* or other hosts in close proximity (Durán et al., 2008). Inoculation trials have shown that the pathogen could infect other *Pinus* species and their hybrids. Susceptibility was seen in *Pinus arizonica* Engelm.*, P. attenuata, Pinus durangensis* Martínez, *P. muricata* D. Don*, P. ponderosa* and *P. rigida* P. Mill.*,* whereas *P. elliottii, P. pinaster, P. strobus* L. and *P. taeda* were shown to be resistant (Ahumada et al., 2013; Widmer and Dodge, 2019).

Symptoms for other hosts such as *P. patula*, *Pinus greggii* Engelm. Ex Parl. and their different hybrids varied considerably (Ahumada et al., 2013). Based on these studies it is evident that pine species closely related to *P. radiata* and native to North and Central America are susceptible to the disease. Therefore, the host range of the pathogen is larger than previously thought but disease development is most likely dependant on the environmental conditions.

4.7.4 Biology and symptoms

The seasonal occurrence of DFP is linked to rainfall patterns during the cold weather of winter. Sporangia at the stomata are the main propagules of infection,

releasing zoospores during rainy periods to aid dissemination (Fig. 7E) (Ahumada et al., 2013). Sporangia are mainly observed on the surface of the needle and are generally absent when the fungus is grown on media (Fig. 7D) (Durán et al., 2008).

Infection occurs on lower branches of the crown during late spring or autumn. Disease symptoms are seen in early winter where resinous bands appear at the site of infection (Fig. 7B). Needles die from the base and discolour grey, giving the tree a scorched appearance (Fig. 7A). Infection at the base of the needle results in resin production, killing the surrounding cambial cells and causing brownish-red discolouration of the vascular tissue (Fig. 7C) (Durán et al., 2008).

Needles may be cast, and continuous defoliation accompanied by secondary pathogens could eventually kill the tree (Durán et al., 2008). In young trees, infection near the fascicle may produce large amounts of resin that causes severe necrosis of the cambium. Cankers begin to develop below the affected tissue and the terminal shoot of the tree begins to wilt and eventually dies (Durán et al., 2008).

4.7.5 Future research

Phytophthora pinifolia severely impacted *P. radiata* plantations in Chile and, given the popularity of this *Pinus* spp. in commercial forestry in the Southern Hemisphere, it is important to determine where the pathogen originated and how it may have been introduced (Durán et al., 2010; Ahumada et al., 2012). The genome of the pathogen has been sequenced and molecular tools have been developed which may aid in this (Durán et al., 2009; Feau et al., 2016). The pathogen has a potentially larger host range than initially thought and using molecular techniques for screening at tentative pathways of introduction is crucial to prevent the accidental introduction into other non-native, commercial plantations.

4.8 Red needle cast

4.8.1 Introduction and taxonomy

Red needle cast was first reported in New Zealand in 2005 when distinct lesions different from any other known pathogens were observed on the needles of *P. radiata* (Dick et al., 2014). Severe outbreaks were later reported in 2008 in the same compartment and isolations made from the lesions yielded a fungus with morphology similar to *Phytophthora* species.

Coincidentally, DNA sequence analyses revealed a new species which falls within ITS Clade III of the *Phytophthora, Phytophthora pluvialis* Reeser, W. Sutton &

E.M. Hansen, described from a stream in southwestern Oregon, USA (Cooke et al., 2000; Reeser et al., 2013). Dick et al. (2014) later found that the unknown species isolated from *P. radiata* in New Zealand is identical to the oomycete described from the USA and is the causal agent of Red needle cast observed in the country. Since its discovery in Oregon, the pathogen has spread to other regions along the west coast of the United States and has recently been reported in southwest England, United Kingdom (Fraser et al., 2020; IPPC, 2021; Tabima et al., 2021).

4.8.2 Distribution and host range

Phytophthora pluvialis has been isolated from a number of different conifer hosts. In the USA, the pathogen has been isolated from canopy drip in forest of mixed tanoak (*Notholithocarpus densiflorus* [Hook. & Arn.] Manos, Cannon, & Oh) and Douglas-fir (*Pseudotsuga menziesii* [Mirbel] Franco) but does not seem to result in clear disease symptoms as is reported on *P. radiata* in New Zealand (Reeser et al., 2013). The pathogen has been reported causing needle chlorosis, loss and crown thinning in Douglas fir planted in close proximity to infected *P. radiata* plots in New Zealand (Hansen et al., 2015). Additionally, the pathogen has also been isolated from *P. patula* and *P. strobus* in New Zealand (Tabima et al., 2021). In the UK, the pathogen was reported on Douglas fir and a strand of Western hemlock (*Tsuga heterophylla* [Raf.] Sarg.), resulting in severe disease symptoms in the latter (IPPC, 2021). The ability of *P. pluvialis* to rapidly diversify and infect other hosts, even in New Zealand where the population is seemingly clonal, suggests that this species has a larger host range than presumed (Hansen et al., 2017; Tabima et al., 2021).

The genome of *P. pluvialis* has recently been sequenced (Studholme et al., 2016) and single nucleotide polymorphisms were used to determine the genetic diversity of the populations in USA and New Zealand. Results indicate that the population in the USA is highly diverse and that the pathogen was introduced into New Zealand from the USA where the pathogen is most likely native or naturalised (Brar et al., 2018; Tabima et al., 2021). This is supported by the sudden onset of red needle cast observed in New Zealand where the conditions are conducive for clonal expansion and further disease outbreaks (Keriö et al., 2019; Tabima et al., 2021).

4.8.3 Biology and symptoms

Symptoms of Red needle cast are mainly seen in older trees but may appear in young trees if in close proximity to a severely diseased older stand (Fig. 8A) (Dick et al., 2014). Initially, water-dispersed propagules infect needles at the base and

produce olive-green to khaki lesions with transverse, resinous, black bands similar to *P. pinifolia* (Fig. 8B-C) (Reeser et al., 2015). These lesions are common in late autumn or winter after several days of rain are followed by optimum temperatures (Dick et al., 2014; Fraser et al., 2020). Lesions expand and coalesce, discolouring the needle yellow and later reddish-brown, eventually resulting in early senescence. The reddish-brown foliage is mainly seen in the lower half of the crown, but can cover the entire crown in severe cases, especially when conditions are wet (Dick et al., 2014).

Needles are cast relatively easily and by late spring many trees are left defoliated (Dick et al., 2014; Scott and Williams, 2014). New foliage remains unaffected by the pathogen, giving the trees an overall healthy appearance at the beginning of summer (Dick et al., 2014). However, repeated defoliation can result in an overall yield loss in *P. radiata* plantations (Ganley et al., 2014). During harsh conditions the fungus survives in the soil in the form of oogonia (Fig. 8E), structures which can be seen when the pathogen is plated onto carrot media (Fig. 8D) (Reeser et al., 2013; Keriö et al., 2019).

4.8.4 Future research

The exact pathway with which the pathogen entered New Zealand is unknown. However, given that the pathogen is unable to survive on bark and logs, but can infect foliage and roots, narrows down the possibilities of tentative pathways (Dick et al., 2014; Hood et al., 2014; Scott et al., 2019). A species-specific PCR assay has been developed for *P. pluvialis* which will play a vital role in developing quarantine regulations to prevent the introduction of this pathogen into any other non-native plantations in the Southern Hemisphere (McDougal et al., 2018).

5. CONCLUSION

There is a long history of *Pinus* spp. being introduced into the Southern Hemisphere to establish commercial plantations to satisfy the demand for wood and wood products. Several *Pinus* spp. were moved between different continents to determine which of these would be better suited to the climate in the Southern Hemisphere. However, *Pinus* spp. can harbour many microorganisms which have inadvertently been introduced into the Southern Hemisphere, along with seed and germplasm, and are now causing disease.

Traditionally, the causal agent of an outbreak was characterised based on its fruiting structures, ecological niches and the disease symptoms which manifest on the

needles. With the advent of DNA sequencing and phylogenetic analyses, it was found that several of these contain cryptic taxa, and this is evident in the history of the needle pathogens discussed in this literature review. In some instances, such as with the outbreaks of Diplodia shoot and tip blight, the causal agents have been well-studied. However, overall, there is still a lack of sampling and molecular confirmation of foliar pathogens of *Pinus* spp. in the Southern Hemisphere. Of the 96 reports of these pathogens on commercially grown *Pinus* spp. in the Southern Hemisphere, the causal agents have only been confirmed using molecular techniques in 35 cases. It is important to validate historical reports to determine the movement of these pathogens and to determine whether any new incursions may have occurred.

Next-generation sequencing will facilitate an appropriate and rapid response to plant pathogens (Hamelin, 2012). Genome sequences can facilitate the development of effective diagnostic tools to resolve species identity, determine the geographic origin and route of introduction of the pathogens and to monitor the success of management strategies against disease outbreaks. Tools such as these have proven useful to reduce the impact pathogens, such as *D. septosporum*, have had on commercial plantations in the Southern Hemisphere in the past.

Climate change will also play a role in the future as it impacts the evolutionary behaviour of pathogens and *Pinus* spp., altering the incidence and severity of disease outbreaks (Garrett et al., 2006). Future climate conditions show that there will be a redistribution of pathogens along the geographical landscape due to changes in water and heat stress. Redistribution along the warmer climates has shown to be negative and in regions such as Africa and Australia, the reduction in precipitation in the winter rainfall areas may result in moisture stress for foliar pathogens. In colder climates, a positive expansion is predicted due to a reduction in cold stress and warmer winter temperatures (Coakley et al., 1999; Garrett et al., 2006; Watt et al., 2011; Woods et al., 2016). It is important to have an adequate understanding of the biology of the pathogen, host and their interactions in order to make accurate predictions to guide management strategies in the commercial forestry industry (Garrett et al., 2011; Watt et al., 2012).

Commercial plantations of non-native *Pinus* spp. will continue being important to countries in the Southern Hemisphere in the future. Even though quarantine measures are implemented, new introductions keep occurring and these plantations will remain under threat. This was illustrated by the recent outbreak of a needle

disease, caused by *L. pharomachri,* on the Mesoamerican *Pinus* spp. introduced into Colombia. These *Pinus* spp. are becoming increasingly important for commercial forestry in some countries in the Southern Hemisphere, and quarantine measures will need to accommodate this new threat. It is evident that many knowledge gaps still remain and will need to be the focus of future work to determine the implications these pathogens may have on commercial plantations in the future.

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7. TABLES

Table 1. The geographic distribution of foliar pathogens on *Pinus* spp. in different countries of the Southern Hemisphere including the earliest date the disease was recorded and whether the causal agent has been verified using molecular techniques.

Pathogen	Phylum	Continent	Country	Earliest report	Reference	Molecular Verification (*)	Reference
Brown needle disease							
Pseudocercospora pini-densiflorae	*Ascomycota*	Africa	Eswatini	Unknown	Ivory (1994)		
			Kenya	1962	Evans (1984)		
			Madagascar	1983	Ivory (1994)		
			Malawi	Unknown	IMI 359224[a]		
			South Africa	1984	Ivory and Wingfield (1986)		
			Tanzania	1960	Gill (1963)		
			Zimbabwe	1965	Ivory (1994)		
			Zambia	1984	Evans (1984)		
		Oceania	Papua New Guinea	Unknown	Ivory (1994)		
Dothistroma needle blight							
Dothistroma septosporum	*Ascomycota*	Africa	Eswatini	1967	Gibson (1972)		

	Kenya	1960	Gibson et al. (1964)	*	Barnes et al. (2004)
	Malawi	1961	Bates (1963)		
	South Africa	1965	Gibson (1972)	*	Barnes et al. (2004)
	Tanzania	1957	Gibson et al. (1964)		
	Uganda	1964	Gibson (1972)		
	Zambia	1994	Ivory (1994)		
	Zimbabwe	1943	Gibson et al. (1964)		
Oceania	Australia	1975	Edwards and Walker (1978)	*	Barnes et al. (2004)
	Papua New Guinea	1997	EPPO (2015)		
	New Zealand	1964	Gibson (1972)	*	Barnes et al. (2004)
South America	Argentina	1968	(Fresa, 1968)		
	Bolivia	1995	IMI 367865[a]		
	Brazil	1969	Figueiredo and	*	Groenewald et al. (2007)

					Namekata (1969)		
			Chile	1965	Dubin and Staley (1966)	*	Barnes et al. (2004)
			Colombia	2008	Rodas et al. (2016)	*	Rodas et al. (2016)
			Ecuador	1982	Evans and Oleas (1983)	*	Barnes et al. (2004)
			Peru	1979	Gibson (1979)		
			Uruguay	1967	(Peterson, 1969)		
Brown spot needle blight							
Lecanosticta acicola	*Ascomycota*	South America	Colombia	1980	Gibson (1980)	*	Janoušek et al. (2016)
Lecanosticta pharomachri	*Ascomycota*	South America	Colombia	2018	Theron et al. (2022)	*	Theron et al. (2022)
Cyclaneusma needle cast							
Cyclaneusma minus	*Ascomycota*	Africa	Kenya	1946	Millar and Minter (1980)	*	Prihatini et al. (2014)
			Malawi	Unknown	Millar and Minter (1980)		

			South Africa	1986	IMI 314466[a]		
			Tanzania	1962	Lundquist (1986)		
		Oceania	Australia	1955	Choi and Simpson (1991)	*	Prihatini et al. (2014)
			New Zealand	1952	Gilmour (1959)	*	Prihatini et al. (2014)
		South America	Argentina	Unknown	Rajchenberg et al. (1995)		
			Ecuador	Unknown	Millar and Minter (1980)		
			Chile	1971	Butin (1973)	*	Vu et al. (2019)
			Colombia	Unknown	Gibson (1979)		
			Uruguay	Unknown	Millar and Minter (1980)		
Cyclaneusma niveum	*Ascomycota*	Africa	Eswatini	1972	IMI 165512[a]		
			Kenya	1961	Nattrass (1961)		
			Malawi	1972	Peregrine and Siddiqui (1972)		

			South Africa	Unknown	Doidge (1950)		
		Oceania	Australia	1962	Sampson and Walker (1982)		
Lophodermium needle cast							
Lophodermium australe	*Ascomycota*	Africa	Kenya	Unknown	Minter (1981)		
			Malawi	1991	IMI 347117[a]		
			South Africa	1982-1983	Roux and Lundquist (1984)		
			Tanzania	1984	IMI 285543[a]		
			Uganda	1984	IMI 291934[a]		
			Zambia	1974	MI 193278[a]		
			Zimbabwe	Unknown	IMI 53463[a]		
		Oceania	Australia	1973	IMI 179652[a]		
			Uruguay	2011	Alonso et al. (2011)		
Lophodermium conigenum	*Ascomycota*	Oceania	Australia	2001	Simpson and Grgurinovic (2004)	*	Prihatini et al. (2015)
			New Zealand	1976	IMI 337982[a]	*	Ortiz-Garcia et al. (2003)

		South America	Chile	Unknown	Butin and Peredo (1986)		
Lophodermium indianum	*Ascomycota*	Africa	South Africa	1982-1983	Roux and Lundquist (1984)		
Lophodermium molitoris	*Ascomycota*	Oceania	New Zealand	Unknown	Johnston et al. (2003)	*	Johnston et al. (2003)
Lophodermium pinastri	*Ascomycota*	Africa	Eswatini	1972	IMI 165512[a]		
			Malawi	1964	Corbett (1964)		
			South Africa	1899	Zahn and Neethling (1929)		
			Zambia	Unknown	IMI 197660[a]		
			Zimbabwe	1961	IMI 91124[a]		
		Oceania	Australia	1960	Stahl (1966)	*	Prihatini et al. (2015)
			New Zealand	1953	Rawlings (1955)	*	Johnston et al. (2003)
			Papua New Guinea	1976	IMI 204981[a]		
		South America	Brazil	Unknown	Mendes et al. (1998)		

			Chile	Unknown	Butin and Peredo (1986)		
Lophodermium seditiosum	*Ascomycota*	Africa	South Africa	1982-1983	Roux and Lundquist (1984)		
		South America	Chile	1980	Rack (1980)		
Diplodia shoot blight							
Diplodia sapinea	*Ascomycota*	Africa	Eswatini	1975	Wingfield and Knox-Davies (1980)	*	Stanosz et al. (1999)
			Kenya	1967	Howland and Gibson (1969)		
			Lesotho	1985-1986	Swart et al. (1987)	*	Stanosz et al. (1999)
			Madagascar	Unknown	Brunck (1964)	*	Stanosz et al. (1999)
			Malawi	1970	Peregrine and Siddiqui (1972)		
			Mauritius	1968	Orieux and Felix (1968)		

	Mozambique	Unknown	de Carvalho (1948)		
	South Africa	1909	Bancroft (1911)	*	Stanosz et al. (1999)
	Tanzania	1965	Howland and Gibson (1969)	*	Stanosz et al. (1999)
	Uganda	1967	Howland and Gibson (1969)		
	Zambia	1980	Rees and Webber (1988)	*	Stanosz et al. (1999)
	Zimbabwe	Unknown	Whiteside (1966)	*	Stanosz et al. (1999)
Asia	Indonesia	Unknown	de Wet et al. (2000)	*	de Wet et al. (2000)
Oceania	Australia	Unknown	Eldridge (1957)	*	Stanosz et al. (1999)
	New Zealand	1936	Birch (1936)	*	Stanosz et al. (1999)
South America	Argentina	1946	Saravi (1950)	*	Bihon et al. (2012a)
	Brazil	±1940	May (1964)	*	Corrêa et al. (2009)

			Chile	Unknown	Spaulding (1961)	*	Wu et al. (2007)
			Paraguay	Unknown	Sanchez (1967)		
			Uruguay	Unknown	Bettucci et al. (2004)	*	Bettucci et al. (2004)
Diplodia scrobiculata	*Ascomycota*	Africa	South Africa	2011	Bihon et al. (2011)	*	Bihon et al. (2011b)
Daño Foliar del Pino							
Phytophthora pinifolia	*Oomycota*	South America	Chile	2004	Durán et al. (2008)	*	Durán et al. (2008)
Red needle cast							
Phytophthora pluvialis	*Oomycota*	Oceania	New Zealand	2005	Dick et al. (2014)	*	Dick et al. (2014)

[a] IMI, International Mycological Institute

8. FIGURES

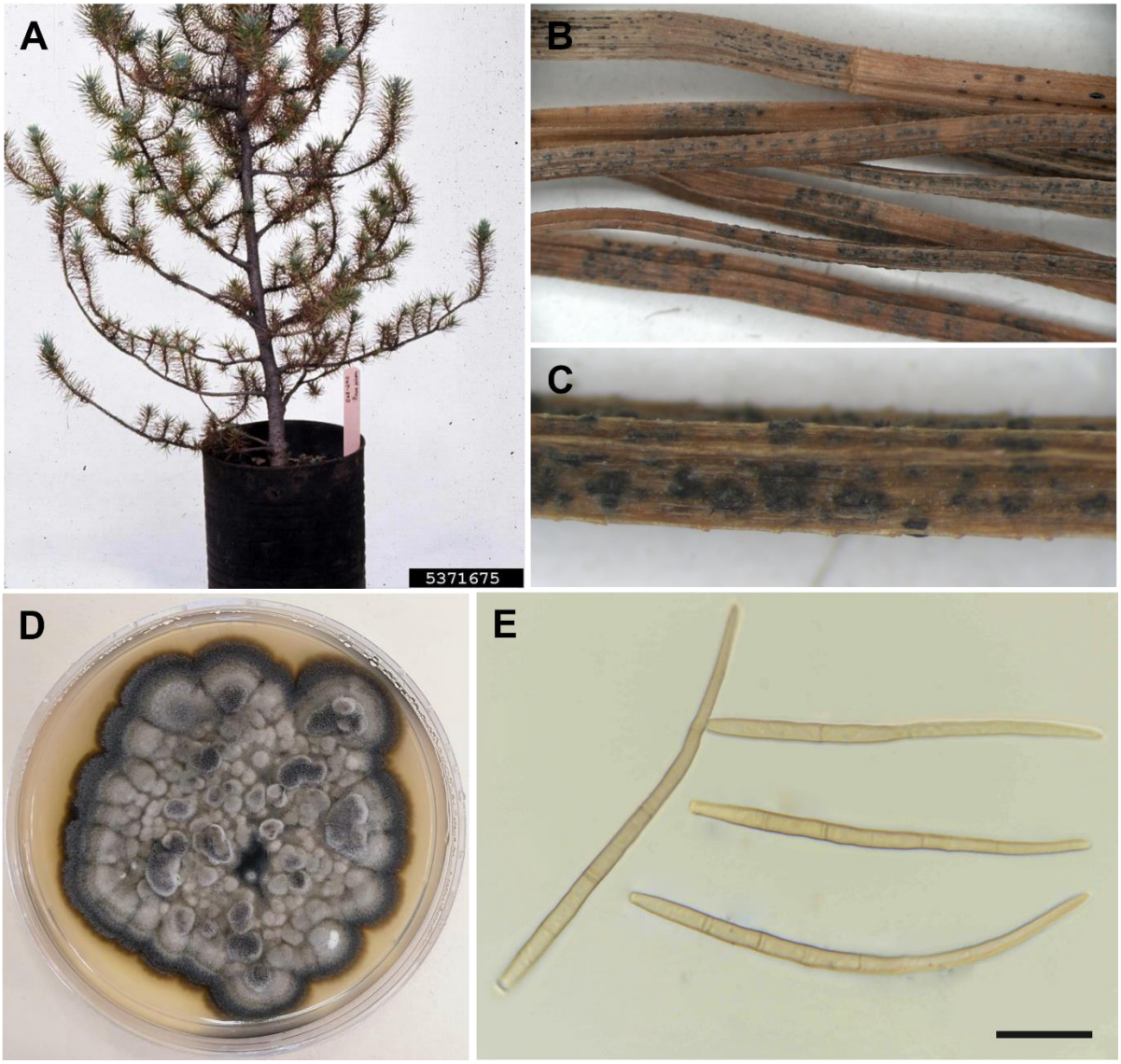

Fig. 1. Brown needle disease caused by *Pseudocercospora pini-densiflora.* **A**, This disease resulted in severe defoliation and death of susceptible *Pinus* spp. (Florida Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Bugwood.org). **B,** Characteristic symptoms observed on the needles include yellow bands that turned brown over time and eventually the needle dies. **C,** Several black conidiomata develop on the needle surface that give the needle a sooty black appearance. **D,** Culture of *Pseudocercospora* sp. isolated from *P. caribaea*. **E,** The conidia of the pathogen are disseminated to adjacent trees by rain-splash. Scale bar = 20µm.

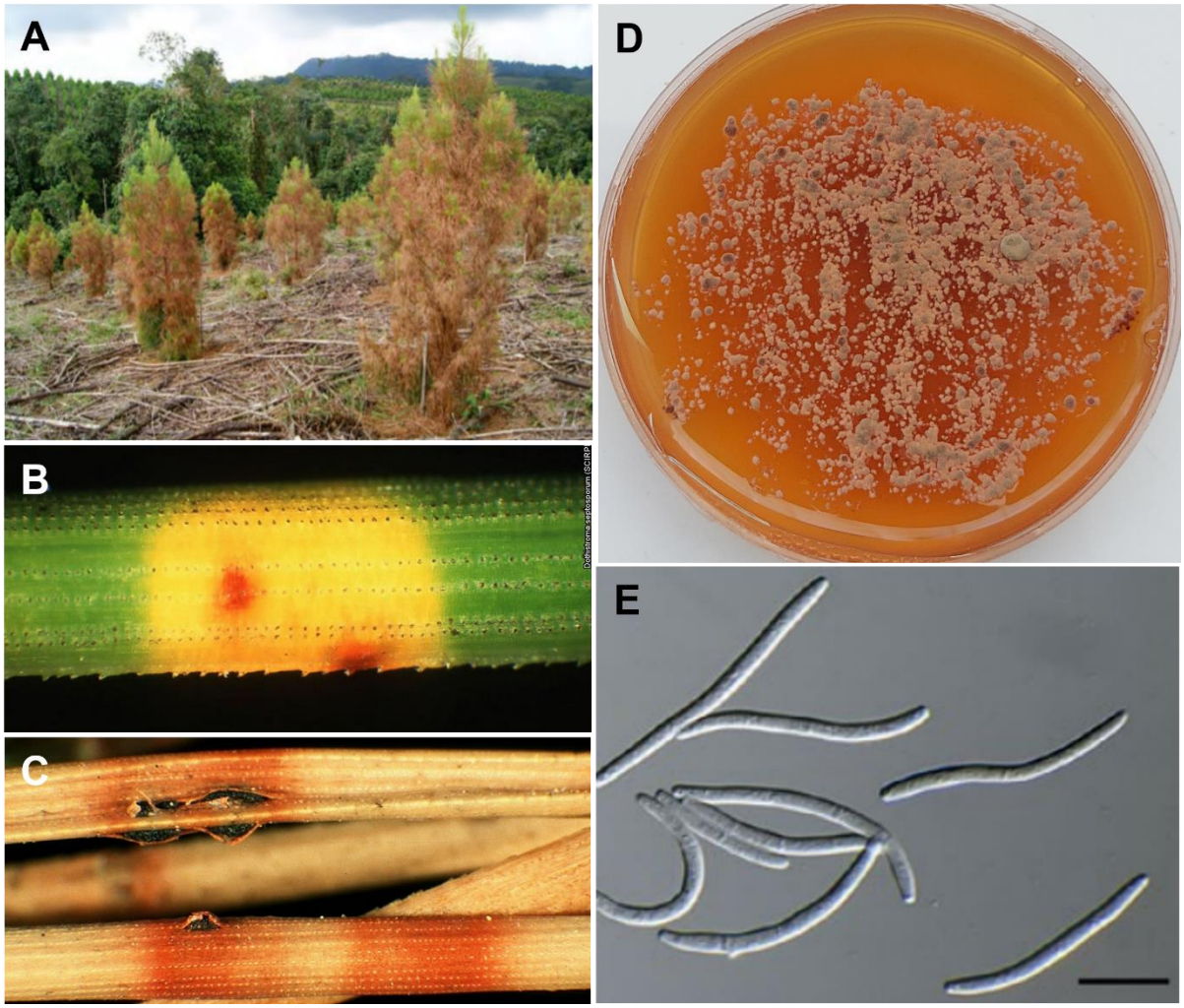

Fig. 2. Dothistroma needle blight is caused by two cryptic species, *Dothistroma septosporum* and *D. pini.* **A,** *Dothistroma septosporum* has been reported in several countries in the Southern Hemisphere and has caused severe damage to commercial plantations of *Pinus* (Rodas and Wingfield, 2020). **B,** At the start of infection, the characteristic discolouration can be seen at the site of infection (EPPO, 2015). **C,** Erumpent conidiomata develop over time, often accompanied by a red band, and eventually the entire needle dies (EPPO, 2015). **D,** The red band seen on the needle surface is caused by the mycotoxin dothistromin, which can be visualised when the species are plated onto media. **E,** The pathogen spreads by conidia which are disseminated to adjacent trees by rain-splash (Barnes et al., 2016). Scale bar: E = 5µm.

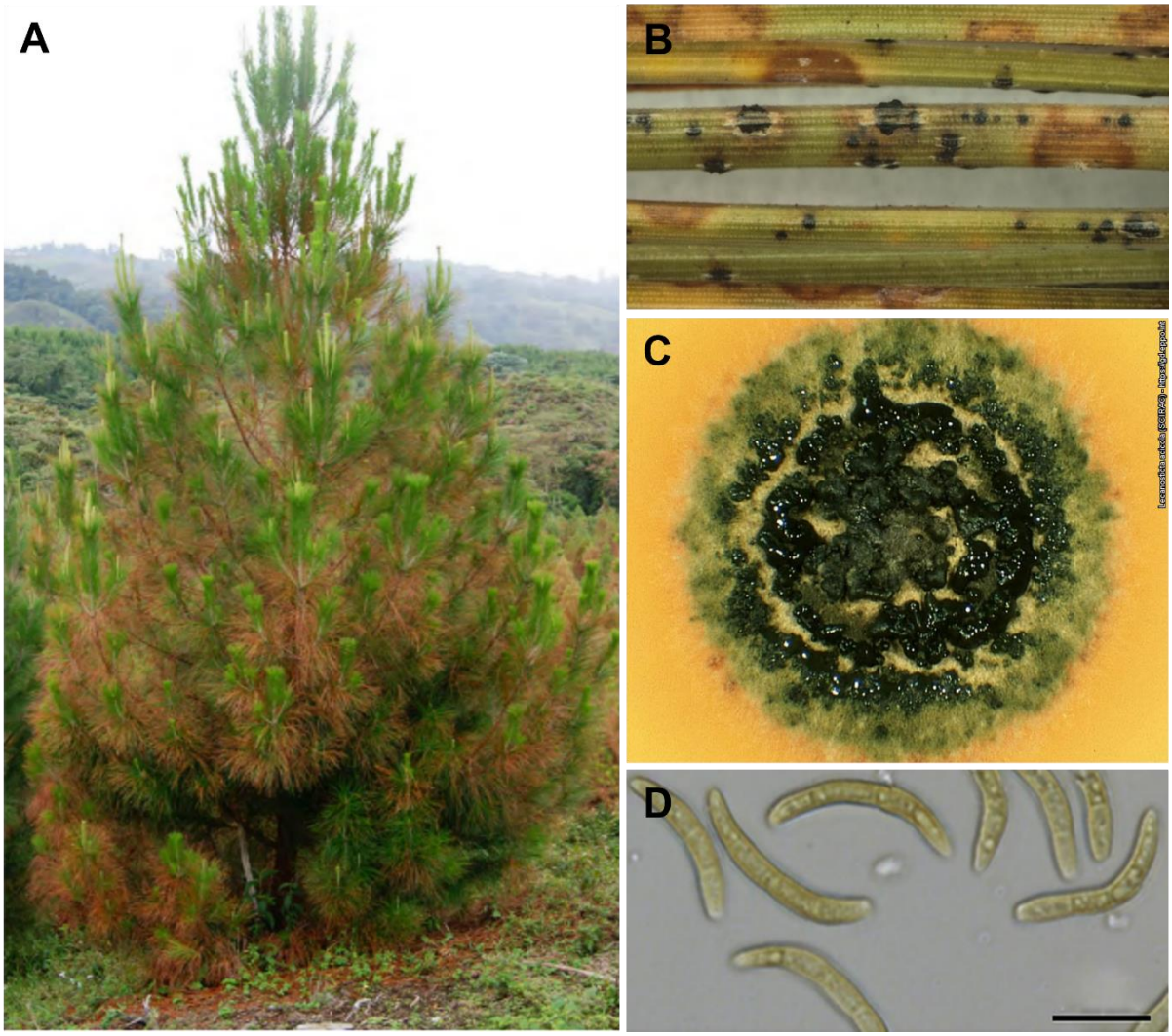

Fig. 3. Brown spot needle blight caused by *Lecanosticta acicola*. **A,** Symptoms are confined to the lower regions of the tree canopy (Rodas and Wingfield, 2020). **B,** The disease results in characteristic brown lesions on the needles with stromata that rupture the needle surface in parallel slits (van der Nest et al., 2019a). **C,** The culture produced by the pathogen is a characteristic grey-green color and the mycotoxin is visualized when it discolors the agar yellow (EPPO, 2001). **D,** The pathogen spreads by conidia which are transferred to adjacent trees by rain-splash (van der Nest et al., 2019a). Scale bar: D = 10µm.

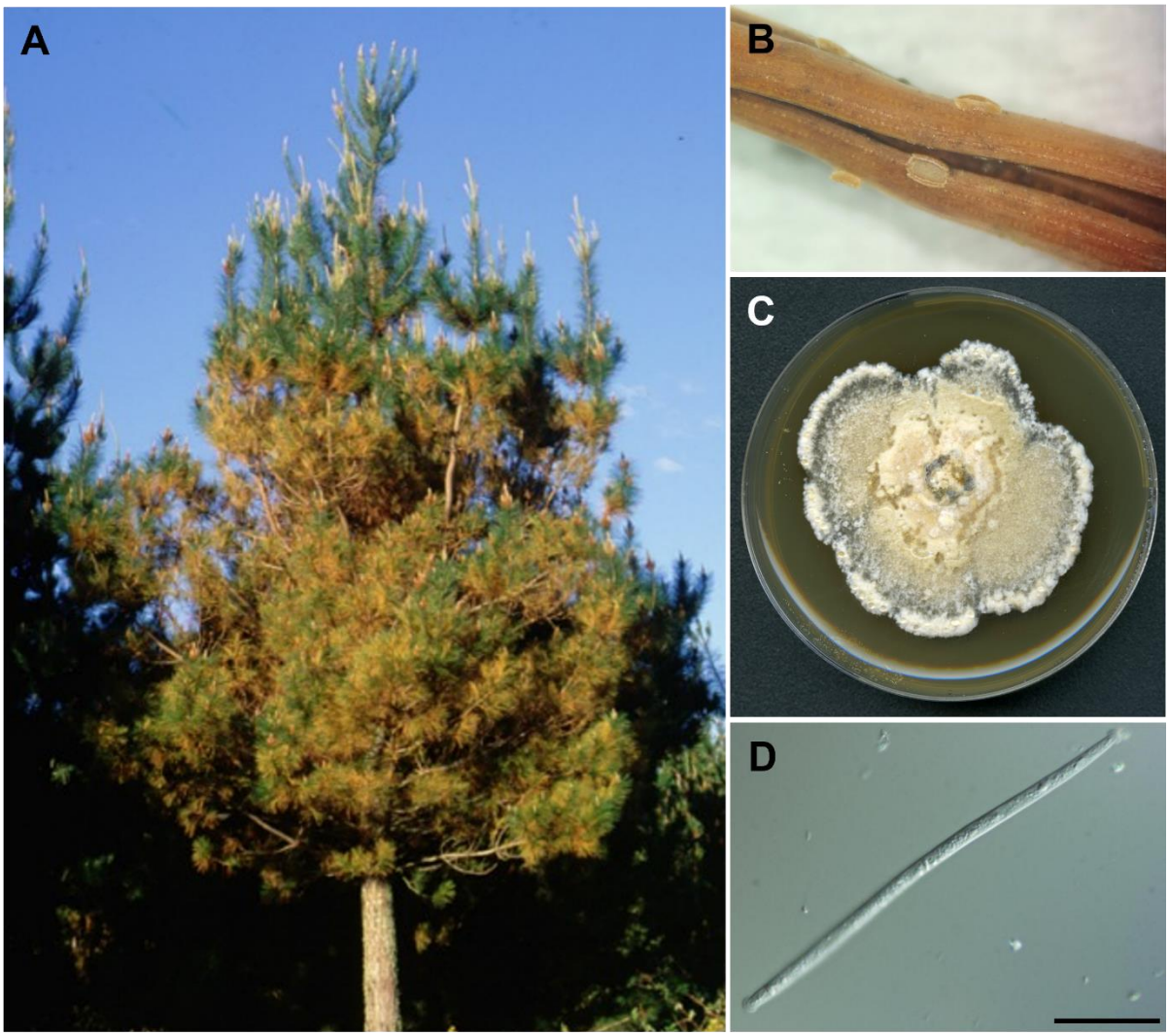

Fig. 4. Cyclaneusma needle cast caused by *Cyclaneusma minus.* **A,** This pathogen has caused extensive damage to *Pinus radiata* plantations in New Zealand (Bulman, 2001). **B,** During periods of high humidity, the characteristic apothecia can be seen on the surface of the needles. **C,** *Cyclaneusma minus* grows easily in culture and in New Zealand and Australia, two different morphotypes have been observed. **D,** Ascospores are released from the apothecia and spread to adjacent trees by rain splash (Watt, University of Maine, Bugwood.org). Scale bar: D = 20 µm.

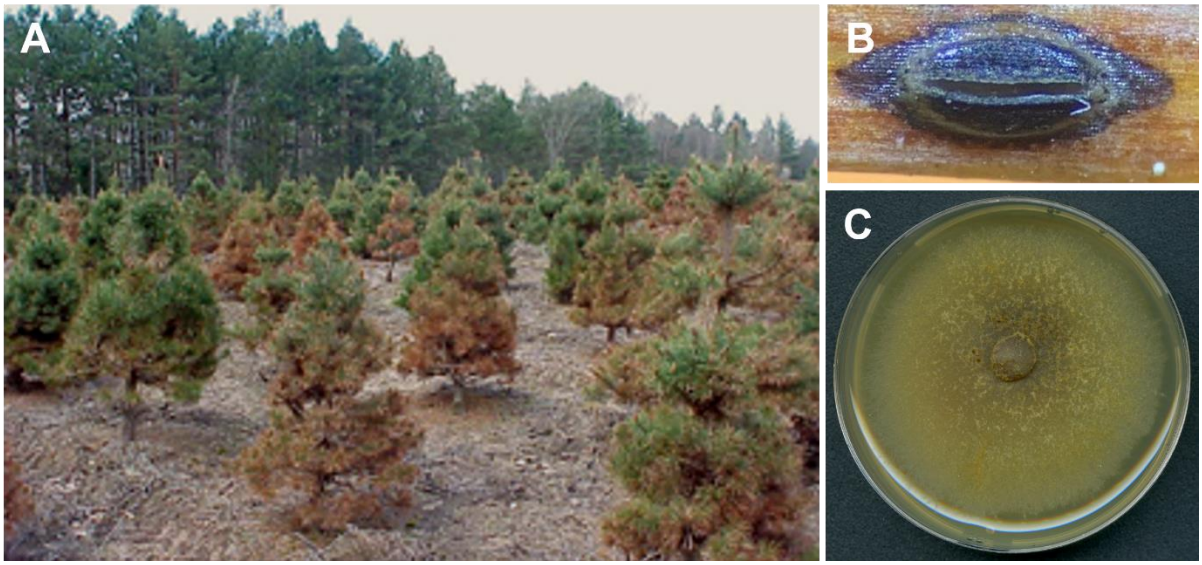

Fig. 5. Lophodermium needle cast caused by *Lophodermium seditiosum.* **A,** The pathogen infects the needles on the lower branches of the trees, resulting in needle death and premature needle cast (O'Donnell & Fulbright, MSU Diagnostic Services, 2010). **B,** The pathogen fruits on the dead needles on the forest floor, forming distinct ascomata that open by a longitudinal slit (Granados, FABI, 2020). **C,** Species of *Lophodermium* grow readily in culture and cultures of *L. seditiosum* are a characteristic tan brown.

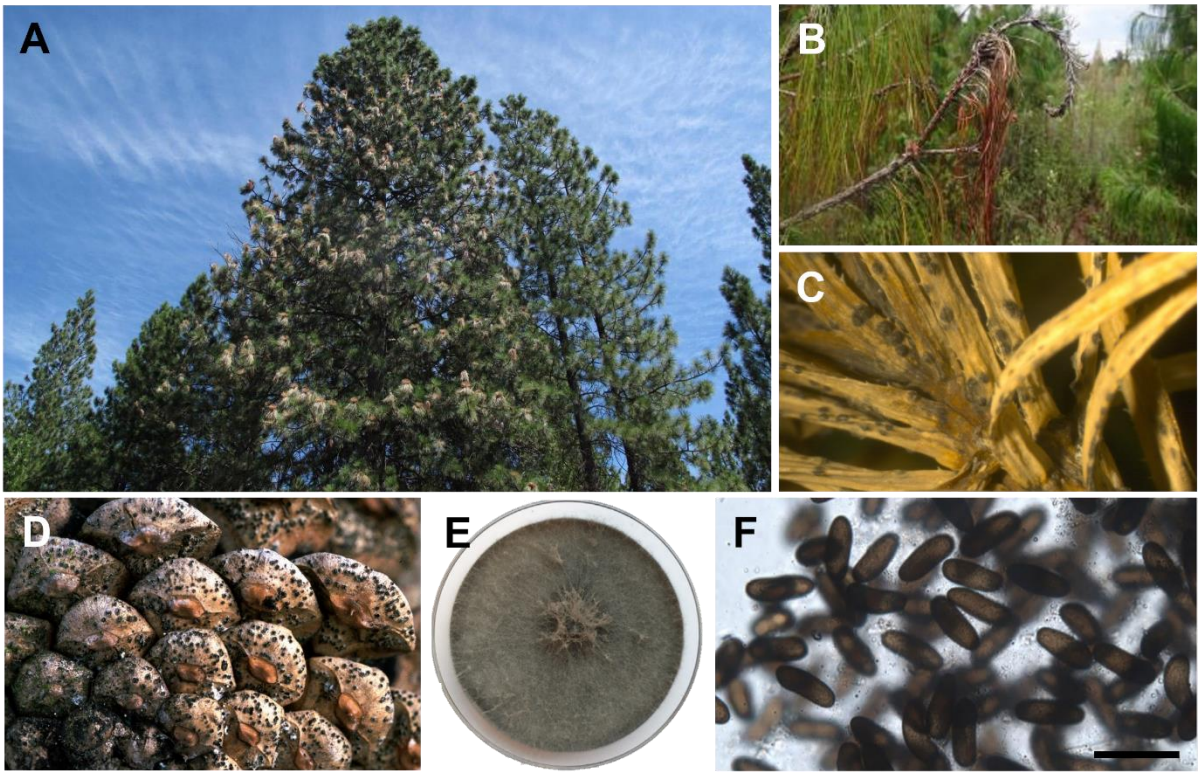

Fig. 6. Tip blight, caused by *Diplodia sapinea.* **A,** This pathogen has a worldwide distribution and can infect young and mature trees (Barnes and Granados, FABI). The pathogen infects the host and causes symptoms as a result of physiological and environmental stresses. **B,** After infection, *D. sapinea* invades the shoots, causing them to dry out and eventually die (Barnes and Granados, FABI). **C and D,** In late summer, small, round, black pycnidia can be seen at the needle base and on cones (Larrson et al., 2021, Kapitola, Central Institute for Supervising and Testing in Agriculture, Bugwood.org). **E,** Grey-brown mycelium is characteristic for *D. sapinea* (Larrson et al, 2021). **F,** The pathogen proliferates in wet conditions and conidia are spread to adjacent trees by wind, rain and insects (Larsson et al., 2021). Scale bar: F = 50µm.

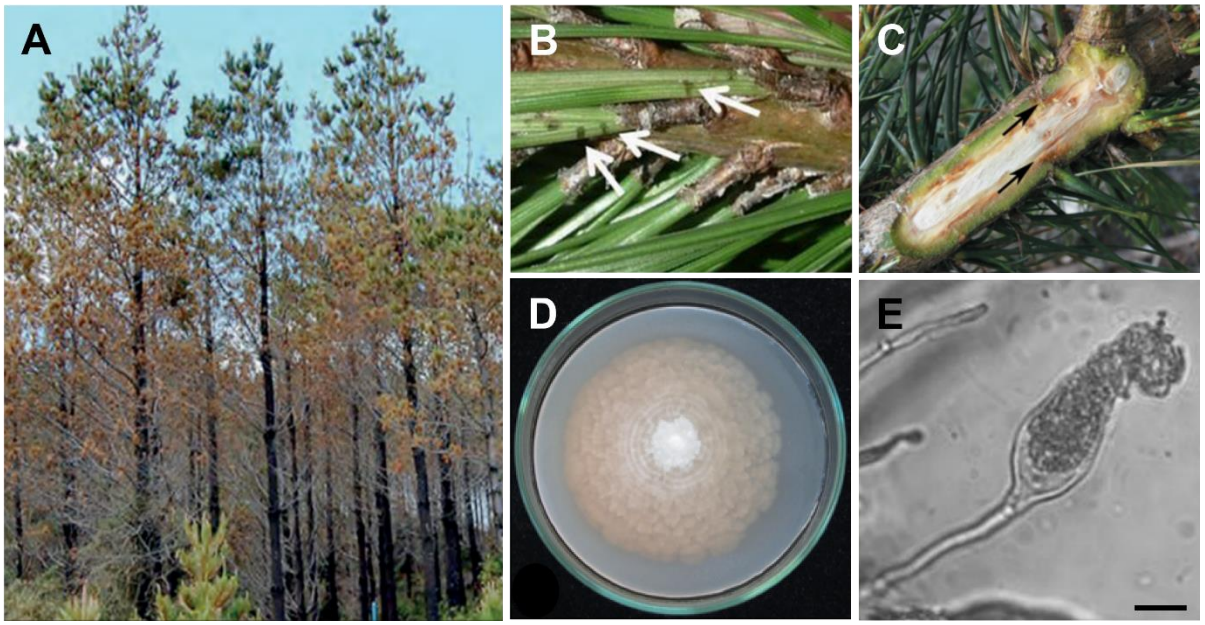

Fig. 7. Daño Foliar del Pino is a disease which first appeared in Chile in 2004 and is caused by the oomycete, *Phytophthora pinifolia.* **A,** This disease resulted in severe defoliation of *Pinus radiata* in these commercial plantations (Ahumada et al., 2012). **B,** The initial symptoms of the disease are resinous bands at the site of infection (Ahumada et al. 2013). **C,** As the disease progresses, the oomycete infects the base of the needle, and this results in brown discolouration of the cambial cells (Durán et al., 2008). **D,** Culture for *Phytophthora pinifolia*. **E,** Zoospores, the main propagule used for infection, are released during moist conditions (Durán et al., 2008). Scale bar: E = 20µm.

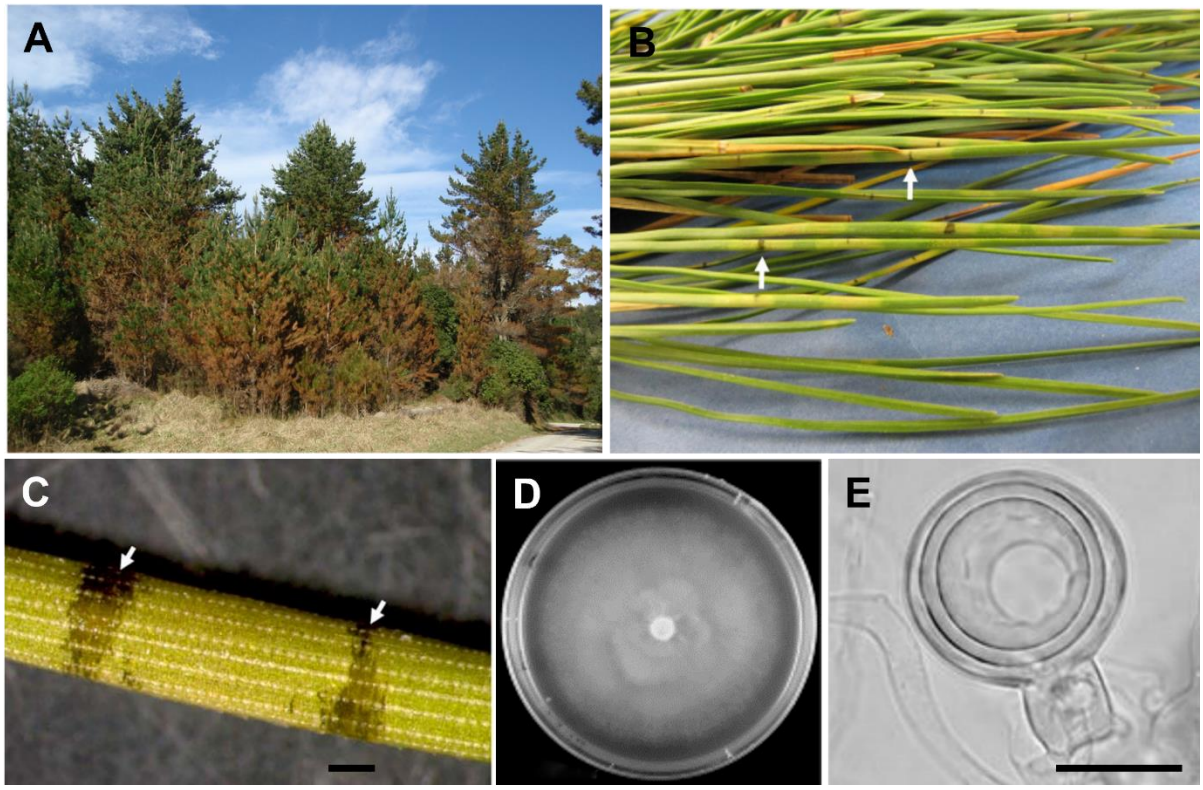

Fig. 8. Red needle cast, caused by *Phytophthora pluvialis*, has only been reported from New Zealand in the Southern Hemisphere. **A,** This pathogen resulted in severe outbreaks on stands of *Pinus radiata* (Dick et al. 2014). **B,** The pathogen spreads by water splash and once it has infected the needle the tissue around the site of infection starts to change yellow and then brown (Dick et al. 2014). **C,** At close inspection, characteristic black, resinous bands can be seen as indicated by the arrows (Dick et al. 2014). **D** *P. pluvialis* can be grown on media and **E,** oogonia may be observed (Hansen et al. 2017, Reeser et al. 2013). Scale bar: C = 0,5mm, E = 20µm.

CHAPTER 2

Lecanosticta pharomachri and its newly discovered sexual state causing serious needle disease of *Pinus* spp. in Colombia

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e-Xtra*

Lecanosticta pharomachri and Its Newly Discovered Sexual State Causing a Serious Needle Disease of *Pinus* spp. in Colombia

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Abstract

Brown spot needle blight (BSNB), caused by the fungal pathogen *Lecanosticta acicola*, is a well-known disease of *Pinus* spp. in several northern hemisphere countries. In the southern hemisphere, this disease has been reported only in Colombia and, apart from a single report of severe defoliation of *Pinus radiata* plantations in the early 1980s, has not caused serious damage in this country. An outbreak of a disease resembling BSNB on Mesoamerican *Pinus* spp. grown in Colombia has raised concern that *L. acicola* may have reemerged as a pathogen. DNA sequence-based analyses for the internal transcribed spacers, translation elongation factor 1-α and RNA polymerase II second largest subunit regions showed that the outbreaks were caused by *L. pharomachri*, a species distinct from, but closely related to, *L. acicola.* The discovery of *L. pharomachri* in Colombia is the first incidence of the pathogen causing a serious disease problem and the first occurrence on the hosts *P. patula* and *P. maximinoi*. A sexual state for *L. pharomachri* was discovered for the first time, and the description of the species has thus been emended.

Keywords: fungi, Mycosphaerellaceae, needle blight, pathogen detection, pine disease, sexual state, tree health

Lecanosticta acicola (Thüm.) Syd. (Mycosphaerellaceae) is an Ascomycete fungus that causes the disease commonly known as brown spot needle blight (BSNB) on *Pinus* spp. BSNB has been reported on 54 different *Pinus* hosts (Mesanza et al. 2021b; van der Nest et al. 2019a), and in severe cases, infection can cause defoliation and tree death (Chapman 1926). *L. acicola* is widespread in the northern hemisphere (van der Nest et al. 2019a) and is best known for a serious outbreak of disease on longleaf pine (*P. palustris* Mill.) in the southeastern United States (Siggers 1932). The severity of the disease caused by this pathogen has resulted in it having a high level of quarantine status in various countries of the world (https://gd.eppo.int/taxon/SCIRAC/categorization).

Mesoamerica is the only region of the world where all nine species of *Lecanosticta* occur, and in this region, they infect native *Pinus* spp. (van der Nest et al. 2019b). It is consequently assumed that this is also the area of origin of these fungi. *L. acicola* is the type species of the genus, and it is mainly found in the northern hemisphere, including Mexico. *L. gloeospora* H.C. Evans and *L. longispora* Marm. were first described from Mexico based on morphology (Evans 1984; Marmolejo 2000) and were later validated based on DNA sequence data (Quaedvlieg et al. 2012). Isolates previously characterized as *L. acicola* from Central America based on morphology were found to represent a species complex, and the novel species were described as *L. brevispora* Quaedvl. & Crous and *L. guatemalensis* Quaedvl. & Crous from Mexico and Guatemala, respectively (Quaedvlieg et al. 2012). In a 2019 study, van der Nest et al. (2019b) described the new species, *L. jani* van der Nest, M.J. Wingf. & I. Barnes from Guatemala and Nicaragua; *L. pharomachri* van der Nest, M.J. Wingf. & I. Barnes from Guatemala and Honduras; *L. tecunumanii* van der Nest, M.J. Wingf. & I. Barnes from Guatemala; and *L. variabilis* van der Nest, M.J. Wingf. & I. Barnes from Guatemala and Honduras. Although there are some minor morphological differences that can be used to distinguish between the species (Quaedvlieg et al. 2012; van der Nest et al. 2019b), they are highly variable and thus provide equivocal results. This, together with the similar disease symptoms associated with infections, necessitates the use of DNA sequence data for accurate species identification (van der Nest et al. 2019b).

L. acicola is the only species in the genus that has been reported as a serious pathogen anywhere in the world. Initial infection causes yellow to gray-green spots on the needles at the site of infection, which turn brown over time and may be surrounded by a chlorotic halo. As infection progresses, the needles die from the apex to the base, and these are eventually cast (Hedgcock 1929; Skilling and Nicholls 1974). Symptoms of BSNB are often confused with those of Dothistroma needle blight (DNB) caused by *Dothistroma septosporum* (Dorogin) M. Morelet and *D. pini* Hulbary (Barnes et al. 2016). Importantly, the characteristic red banding produced by the *Dothistroma* pathogens can be absent or darker in color, giving a false impression of BSNB infection (Barnes et al. 2004).

At the end of the growing season, dark olivaceous to black erumpent asexual conidiomata develop on living or dead needles, and these facilitate overwintering (Siggers 1944). The sexual state of *L. acicola* is extremely rare, having only been reported in southern regions of the United States, parts of Central America (now unconfirmed; see van der Nest et al. 2019a), and Colombia (Evans 1984; Henry 1954; Kais 1971; Siggers 1944), and in 2021, on *P. radiata* D. Don from Europe (Mesanza et al. 2021a). Ascostromata of *L. acicola* develop primarily on dead needles and forcibly discharge ascospores that are spread over long distances by air currents (Siggers 1939; Wolf and Barbour 1941).

BSNB was first reported in Colombia by Gibson (1980), where it was found on *P. radiata*, *P. elliottii* Engelm., and *P. patula* Schiede ex Schltdl. & Cham. in Antioquia. Later, both the sexual and asexual states of *L. acicola* were reported on *P. radiata* in Albán, Cundinamarca, by Evans (1984). Both these reports relied solely on morphological characteristics for identification. The only confirmation of the presence of *L. acicola* in Colombia arises from two samples isolated from *P. caribaea* Morelet in Villanueva, Casanare, that were verified

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using translation elongation factor 1-α (*TEF*1) sequence data (Janoušek et al. 2016). These two isolates reside in the southern lineage of *L. acicola*, which is one of three distinct clades for the species (Janoušek et al. 2016; van der Nest et al. 2019b). It is consequently possible that *L. acicola* resulted in the severe defoliation of *P. radiata* plantations in Colombia reported by Evans (1984).

There are no native *Pinus* spp. in Colombia, and the commercial forestry sector relies on species that are mostly native to Mexico and Central America. Other than severe damage to *P. patula* caused by *Diplodia sapinea* (Fr.) Fuckel (Rodas and Osorio 2008; Rodas and Wingfield 2020), these Mesoamerican *Pinus* spp. have not been affected by serious needle diseases. This situation changed in 2008 when DNB caused by *Dothistroma septosporum* arose as a serious threat to pine forestry in Colombia and especially to the extensively planted *P. tecunumanii* Eguiluz & J. P. Perry of the low-elevation (LE) population (Rodas et al. 2016). The Mesoamerican species *P. patula*, *P. tecunumanii* of the high-elevation (HE) population, and *P. maximinoi* Moore were most tolerant to infection by the pathogen (Rodas et al. 2016).

Since 2018, symptoms resembling BSNB began to appear on *P. maximinoi*, *P. patula*, and *P. tecunumanii* LE in areas where these species are propagated in Colombia. These new disease outbreaks, specifically on Mesoamerican pine species and in areas where *L. acicola* has not previously been reported, required an identification of the causal pathogen. This was especially considering that various new *Lecanosticta* spp. have been reported from Mesoamerica. The aim of this study was, therefore, to identify the pathogen causing the severe outbreak of BSNB symptoms in Colombia by employing both DNA sequence and morphological data.

Materials and Methods

Sample collection and pathogen isolation. Pine needles from trees ranging from 6 months to 3 years of age with characteristic symptoms of BSNB were collected from a wide distribution of farms across the pine-growing regions of Colombia between January 2018 and February 2020. These were placed in paper envelopes and stored at –80°C to kill surface contaminants and insects (Barnes et al. 2004). Needles bearing characteristic conidiomata were placed in moist chambers for 24 h before aseptically excising these structures. The conidiomata were rolled across the surface of 2% Dothistroma sporulating media (DSM: 5 g of yeast extract, 20 g of malt extract, 17 g of agar per liter of distilled water) supplemented with 100 mg/liter of streptomycin as described by Barnes et al. (2004) to release conidia. After 1 to 2 days, the tips of single germinating conidia were transferred to a new DSM plate and grown at 23°C for 3 to 4 weeks. Ascomata were observed on a single *P. patula* sample, and two structures were aseptically excised from the needles and transferred to DSM to obtain pure cultures. All isolates obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, the University of Pretoria, South Africa.

DNA extraction and PCR amplification. Fungal mycelium was scraped from the surface of the DSM agar in Petri dishes with a sterile blade and lyophilized. Freeze-dried material was pulverized using metal beads for 3 min in a Retsch MM301 mixer mill. DNA was extracted using the method described by Barnes et al. (2001), with minor modification. Tubes containing the pulverized tissue and extraction buffer were first incubated on a heating block for 15 min at 85°C followed by 1 h at 60°C before further processing. PCR amplification was performed for three regions: the internal transcribed spacer (ITS) 1, 2, and 5.8 S rDNA, using primers ITS1 and ITS4 (White et al. 1990), a portion of the *TEF*1 using the primers EF1-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998), and the RNA polymerase II second largest subunit (*RPB*2) using primers RPB2-5f2 (Sung et al. 2007) and RPB2-7cR (Liu et al. 1999). In addition, the ITS and *RPB*2 regions were amplified for the *L. acicola* isolate (CMW37199) previously collected in Colombia (Janoušek et al. 2016).

PCR amplifications were performed in 25 µl reactions containing 30 ng of DNA, 2.5 µl of 10× PCR reaction buffer, 2.5 mM of $MgCl_2$, 400 nM of each of the primers, 200 µM of each dNTP, and 1 U of FastStart *Taq* DNA Polymerase (Roche Diagnostics, Indianapolis, IN) and the difference adjusted with sterile SABAX water (Adcock Ingram, Bryanston, South Africa). PCRs were performed for each primer pair according to the cycling parameters defined by van der Nest et al. (2019b) on an Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). Amplification was confirmed by staining 3 µl of product with 1 µl of GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA) and separating it on a 2% agarose gel at 100 V for 25 min, after which it was visualized under ultraviolet light.

Sequencing. PCR products were cleaned with a 6.65% G-50 Sephadex solution (MilliporeSigma, Billerica, MA) using Centri-Sep columns (Princeton Separations, Freehold, NJ) following the manufacturer's protocols. The forward and reverse reactions were sequenced in 12 µl reaction volumes. The reactions contained 60 to 100 ng of PCR product, 1 µl of BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied BioSystems, Thermo Fisher Scientific), 2 µl of sequencing buffer, and 1 µl of either the forward or reverse primer. Reaction volumes were adjusted using sterile SABAX water and sequenced on the ABI PRISM 3500xl auto-sequencer (Applied Biosystems, Thermo Fisher Scientific) at the sequencing facility of the University of Pretoria. The forward and reverse sequencing reads were imported into CLC Bio Main Workbench v6 (CLC Bio, https://www.qiagenbioinformatics.com/products/clc-main-workbench/) to construct contigs and consensus sequences that were exported for phylogenetic analysis.

Phylogenetic analyses. The *TEF*1 gene region is considered a secondary barcoding locus for the Mycosphaerellaceae because it differentiates between closely related species (Quaedvlieg et al. 2012; van der Nest et al. 2019b). Sequences for the *TEF*1 gene region were consequently used for BLAST (Basic Local Alignment Tool) analysis against the NCBI (National Center for Biotechnology) GenBank nucleotide database (http://www.ncbi.nlm.nih.gov) to provide a preliminary identification of the isolates. Sequences obtained in this study were added to the dataset of van der Nest et al. (2019b), along with those of Janoušek et al. (2016) for *L. acicola* in Colombia, and these were subjected to phylogenetic analyses. Sequences were aligned using the MAFFT v7 online resource (https://mafft.cbrc.jp/alignment/server/) using the default settings, and alignments were imported and manually examined in the Molecular Evolutionary Genetics Analysis (MEGA-X) software (Kumar et al. 2018). Subsequently, the tree topologies were visually compared to confirm congruency and FASconCAT-G (Kück and Longo 2014) was used to establish a combined dataset for downstream analyses. A maximum likelihood (ML) analysis was performed for the individual and combined datasets on the IQ-Tree (Trifinopoulos et al. 2016) Web server (http://iqtree.cibiv.univie.ac.at/) where trees were constructed based on the best ranked model according to the Bayesian information criterion by ModelFinder (Kalyaanamoorthy et al. 2017), and confidence levels were estimated by 1,000 bootstrap inferences. Isolates of *Dothistroma septosporum* (CMW44656) and *Phaeophleospora gregaria* (CMW45434) maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, the University of Pretoria, South Africa (van der Nest et al. 2019b) were used as the outgroup taxa.

A Bayesian inference analysis was performed in MrBayes v3.2.7a (Ronquist et al. 2012) on the datasets by applying the Markov Chain Monte Carlo (MCMC) method to determine the posterior probabilities. Four independent MCMC chains were randomly started for four million generations based on the best substitution model determined by ModelFinder, and trees were sampled every 100 generations. Tracer v1.6 was used to visualize the stationarity and determine the burn-in values (10%) by comparing the log likelihoods. Phylogenetic trees were visualized in FigTree v1.4.4 and edited in Inkscape v0.92.4.

Morphological observations. Morphological characteristics of the sexual and asexual states of the fungus were studied using structures taken directly from *P. patula* and *P. maximinoi* needles, respectively. Microscopic structures were initially mounted on glass slides in water, and this was later replaced with 85% lactic acid. Photographic images were captured, and measurements made using

Nikon microscopes (Nikon Eclipse N*i* and SMZ18) and a camera (Nikon DS-Ri2). To observe the sexual and asexual fruiting structures and their arrangement in the host tissue, dried needles containing fruiting structures were boiled in water for a few seconds. Softened needles were then cut into small pieces, 1 to 2 mm in length, and were subsequently embedded in tissue freezing medium (Leica Biosystems, Buffalo Grove, IL). The pieces were sectioned lengthwise at 10 µm thickness using a freezing microtome (LEICA CM1520) and mounted in 85% lactic acid for observation. Fifty measurements were taken for spores, and a limited number of measurements were taken for other structures depending on their availability. Measurements were described as minimum–maximum (average ± standard deviation).

Results

Fungal isolates and morphological observations. Disease symptoms (Fig. 1) appeared mainly in young plantations on approximately 6-month-old trees, with infection levels of up to 30% of the needles, concentrated in the lower parts of the canopy (Fig. 1A and B). Erumpent, black conidiomata were found clustered together, rupturing the epidermis along the surface of the needles. These structures were especially prominent in *P. maximinoi* (Fig. 1C).

Approximately 30 isolates, tentatively identified as a species of *Lecanosticta* based on culture morphology as described by van der Nest et al. (2019b), were obtained from the needles collected across the commercial pine-growing region of Colombia (Table 1). Of these, 10 were from *P. maximinoi*, 16 were from *P. patula*, and four were from *P. tecunumanii* LE. Culture morphology on DSM varied significantly between the different isolates ranging from olivaceous green with white aerial mycelium to a white mycelial mat with rosy buff edges. All isolates produced a yellow exudate that diffused into the DSM after 4 weeks (Fig. 1D and E).

Phylogenetic analyses. BLASTn analyses against the GenBank database using sequences for the *TEF*1 region tentatively identified all the isolates as *L. pharomachri* with similarity hits ranging

Fig. 1. Symptoms, fruiting structures, and culture morphology of *Lecanosticta pharomachri* from Colombia. **A,** Planted *Pinus patula* and **B,** *P. maximinoi* bearing necrotic needles on 6-month-old trees. **C,** Conidiomata with conidia rupturing the epidermis in a dark olivaceous mass. **D** (CMW56116) and **E** (CMW56122), differences in culture morphology and characteristic yellow exudate observed on Dothistroma sporulating media.

from 94.5 to 97.5% against the ex-type of *L. pharomachri* (Accession No. MK015502.1). A subset of 16 isolates representative of different hosts and regions, and including a single isolate of *L. acicola* previously identified in Colombia (Janoušek et al. 2016), were selected for further phylogenetic analyses. These included an isolate obtained from an ascospore extracted from a sexual structure on *P. patula*. The relative amplicon lengths for the ITS, *TEF*1, and *RPB*2 were 528, 490, and 910 bp, respectively. All sequences generated in this study were deposited in GenBank (Table 1).

Datasets were aligned for the ITS (40 taxa, 672 characters), *TEF*1 (40 taxa, 516 characters), *RPB*2 (40 taxa, 918 characters), and combined (40 taxa, 2,106 characters) regions. ML phylogenetic analyses consistently grouped the isolates from Colombia collected in this study within the *L. pharomachri* clade. The *TEF*1 gene region clearly defined all known *Lecanosticta* spp. and showed that the isolates in this study were genetically different from *L. acicola* previously reported in Colombia (Janoušek et al. 2016). Sequences from the *TEF*1 gene region had the highest intraspecific variation, and isolates of *L. pharomachri* had a branch support of 66% (Fig. 2). The isolates from Colombia defined four unique haplotypes that were different from the haplotypes identified in isolates of this fungus from Central America (van der Nest et al. 2019b). These included 34 variable sites across the four haplotypes. One unique haplotype contained a nine base pair insertion in four of the isolates, including the sequence for the sexual state of the fungus (Fig. 2). Different haplotypes were found for isolates from a single host sample, and there was no correlation between the haplotypes and the area or host from which they were isolated. The ITS region revealed two haplotypes, resulting from a single base pair difference, with a branch support >90% (Supplementary Fig. S1). One haplotype was unique in that it included only two isolates from *P. patula* (Supplementary Fig. S1). The *RPB*2 gene region had the weakest signal. The *RPB*2 sequences had less intraspecific variation than the *TEF*1, and only four variable sites were observed between the isolates from Colombia. Overall, there were six haplotypes for *L. pharomachri*, four unique to Colombia, one identical to *L. pharomachri* isolates from Guatemala (CMW38947 and CMW38974), and one haplotype specific to the ex-type of *L. pharomachri* (Supplementary Fig. S2). Thus, results of the phylogenetic analyses for the combined dataset consistently supported the identification and intraspecific variation of the *L. pharomachri* isolates on different *Pinus* spp. planted across the pine-growing region of Colombia (Supplementary Fig. S3).

Morphological observations. The sexual state of *L. pharomachri* was found only once and on a single sample of *P. patula* collected on the farm Cuprecia, Cauca. The black ascomata were scattered along the length of exclusively dead needles, and cross sections through the embedded ascomata revealed white locules (Fig. 3A to D). Asci contained eight ascospores that were hyaline with rounded apices and had tapered trunks and a median septum (Fig. 3E and F). In contrast, the asexual state occurred abundantly and was isolated from needles on each of the tree hosts, *P. patula*, *P. maximinoi*, and *P. tecunumanii* LE. The conidiomata were black and erumpent and scattered across the length of either green or dead needles (Fig. 4A to I). Conidia that emerged from the conidiophore were subhyaline to light brown and slightly curved, with one to three septa (Fig. 4J to L).

Table 1. Details of the *Lecanosticta* isolates from Colombia used in the morphological and phylogenetic analyses

CMW numbers[a]	Sampling site	*Pinus* host	Collector	GenBank accession numbers[b]		
				ITS	*TEF*1	*RPB*2
L. acicola						
37199	Villanueva, Casanare	*P. caribaea*	C. A. Rodas	MZ540372	KJ938451	MZ675995
L. pharomachri						
53480[c]	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540373	MZ675977	MZ676006
54477	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540380	MZ675978	MZ676007
56107	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540384	MZ675968	MZ675997
56108	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540385	MZ675967	MZ675996
56111[c]	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540386	MZ675979	MZ676008
56116[c]	Roble Bonito, Caldas	*P. patula*	C. A. Rodas	MZ540390	MZ675976	MZ676005
54480[c,d]	Salinas, Cauca	*P. patula*	J. Mendez	MZ540382	MZ675980	MZ676009
54481[d]	Salinas, Cauca	*P. patula*	J. Mendez	MZ540383	MZ675981	MZ676010
56117[c]	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540391	MZ675986	MZ676016
56118	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540392	-	-
56120	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540394	MZ675988	MZ676018
56122	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540396	MZ675989	MZ676019
56123	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540397	MZ675990	MZ676020
56124[c]	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540398	MZ675987	MZ676017
56114	Salinas, Cauca	*P. patula*	C. A. Rodas	MZ540388	MZ675991	MZ676021
56115	Peñas Negras, Cauca	*P. maximinoi*	C. A. Rodas	MZ540389	MZ675993	MZ676024
56113[c]	Peñas Negras, Cauca	*P. patula*	C. A. Rodas	MZ540387	MZ675994	MZ676013
56125	Peñas Negras, Cauca	*P. maximinoi*	C. A. Rodas	MZ540399	-	MZ676022
56121[c,e]	Peñas Negras, Cauca	*P. maximinoi*	C. A. Rodas	MZ540395	MZ675992	MZ676023
54479[c]	Esperanza, Cauca	*P. tecunumanii*	J. Mendez	MZ540381	MZ675982	MZ676011
56193[c]	Casas Bajas, Cauca	*P. tecunumanii*	C. A. Rodas	MZ540402	MZ675983	MZ676012
56119[e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540393	MZ675970	MZ675999
54470[c,e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540375	MZ675975	MZ676004
54471[c,e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540376	MZ675974	MZ676003
54472[c,e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540377	MZ675971	MZ676000
54469[c,e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540374	MZ675972	MZ676001
54473[c,e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540378	MZ675969	MZ675998
54474[e]	Graminea, Caldas	*P. maximinoi*	C. A. Rodas	MZ540379	MZ675973	MZ676002
56190[c]	Versalles, Cauca	*P. tecunumanii*	C. A. Rodas	MZ540400	MZ675984	MZ676014
56191	Versalles, Cauca	*P. tecunumanii*	C. A. Rodas	MZ540401	MZ675985	MZ676015

[a] CMW, culture collection of the Forestry and Agricultural Biotechnology Institute, the University of Pretoria, South Africa.
[b] ITS, internal transcribed spacers; *TEF*1, translation elongation factor 1-α; and *RPB*2, RNA polymerase II second largest subunit.
[c] Isolates used in phylogenetic analyses.
[d] Needle samples used in morphological analyses of the sexual state.
[e] Needle samples used in morphological analyses of the asexual state.

Taxonomy. *L. pharomachri* has previously been described only from culture (van der Nest et al. 2019b). The presence of both the sexual and asexual states of the fungus on pines in Colombia has made it possible to prepare a detailed description of the species directly from needles. The following emended description is, therefore, provided:

***L. pharomachri* van der Nest, M.J. Wingf. & I. Barnes., IMA Fungus. 10. 2019.** *Sexual* (Fig. 3) *Ascostromata* immersed in host tissues, subepidermal or subhypodermal, arranged longitudinally along length of needle, bursting through epidermis at maturity, opening via an ostiole, ostiole reaching surface; stromatic tissues pseudoparenchymatous, composed of thick-walled, brown cells; fertile region multiloculate, locules subglobose to obpyriform in cross section, 82 to 110 × 71 to 94 µm (95.5 ± 9.0 × 84.1 ± 7.4 µm). *Asci* bitunicate, narrowly cylindrical to clavate, eight-spored, 45 to 52 × 5 to 8 (47.8 ± 2.34 × 6.9 ± 0.82 µm). *Ascospores* hyaline, smooth, fusoid, two-celled, straight or slightly curved, septum median or nearly so (supra-median), slightly constricted, 10 to 15 × 3 to 4 µm (13.03 ± 1.26 × 3.25 ± 0.31 µm).

Asexual (Fig. 4) *Conidiomata* acervular, subepidermal, dehiscing epidermis, becoming erumpent when mature, pulvinate, 63 to 275 × 82 to 424 µm (171 ± 58.7 × 223 ± 89.7 µm) in section; basal tissue eustromatic, prosenchymatous or pseudoparenchymatous, composed of subhyaline, thick-walled cells; vegetative hyphae present beyond stromatic structure toward mesophyll, not beyond endodermis, thick-walled, pale brown to brown. *Conidiophores* cylindrical, pale brown, septate, surrounded by mucilaginous substance, 26 to 52 × 3 to 6 (35.3 ± 7.33 × 4.8 ± 0.61 µm). *Conidiogenous cells* holoblastic, cylindrical, hyaline, integrate. *Conidia* fusiform to cylindrical, tapering toward apex and base, straight or slightly curved, 1- to 3-septate, verruculose, subhyaline to pale brown, base truncated, 28 to 49 × 3 to 5 µm (39.5 ± 4.38 × 3.8 ± 0.38 µm).

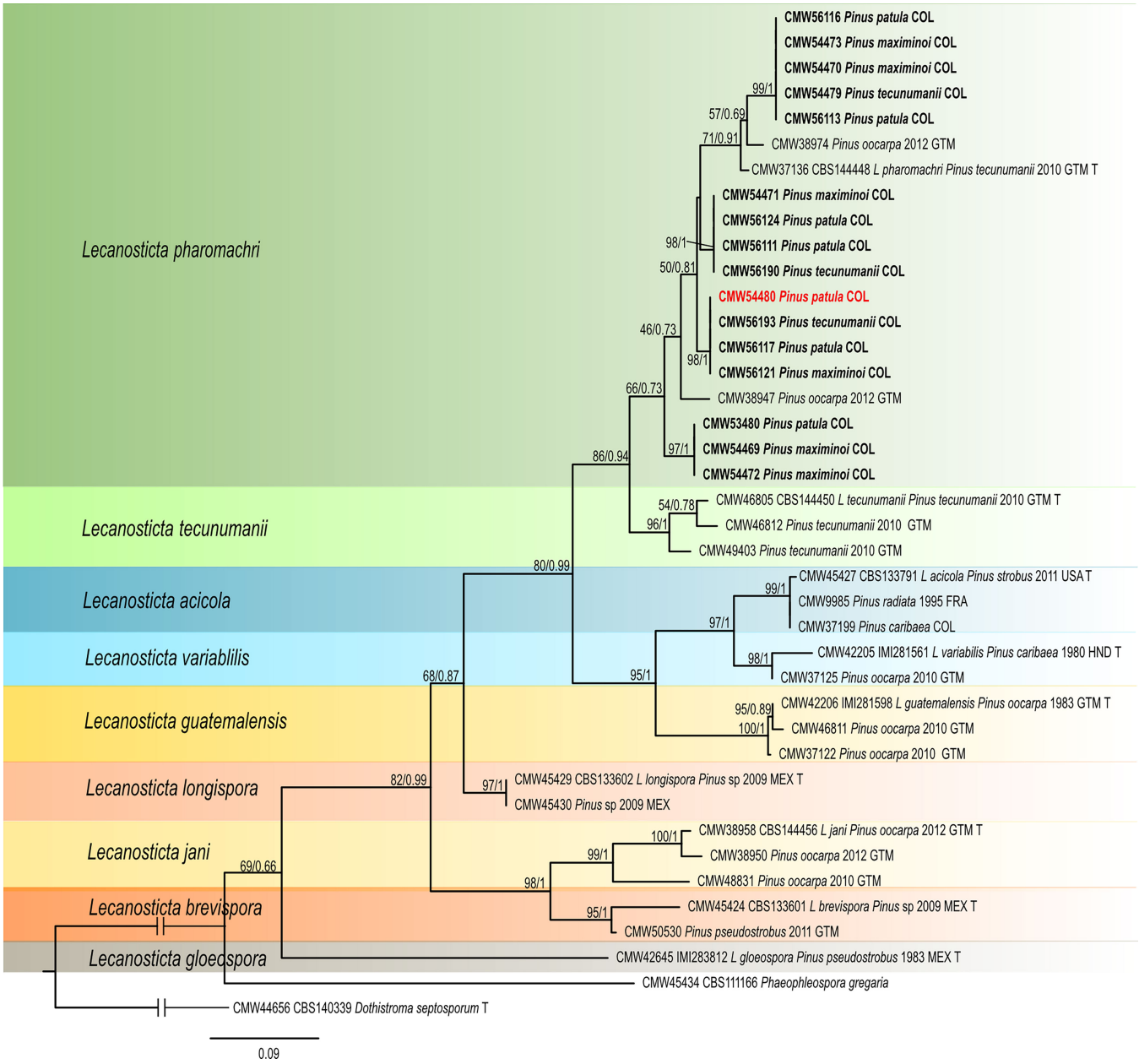

Fig. 2. Maximum likelihood tree based on the translation elongation factor 1-α region for all *Lecanosticta* spp. Isolates generated in this study, indicated in bold, fall within the *L. pharomachri* clade. The sequence from the sexual state of *L. pharomachri* is indicated in red. Ex-type specimens are indicated with a "T," and *Dothistroma septosporum* and *Phaeophleospora gregaria* were used as the outgroup taxa. Numbers at the nodes indicate the bootstrap support (n = 1,000) and posterior probabilities from Bayesian inference, respectively.

Discussion

The results of this study showed that the pine needle pathogen, *L. pharomachri*, has emerged for the first time outside its apparent native range and is causing serious damage to plantation-grown and non-native *Pinus* spp. in Colombia. This pathogen was taxonomically described for the first time in 2019 on native *Pinus* spp. from two countries in Central America (van der Nest et al. 2019b) and has evidently been accidentally introduced into Colombia. It was also found for the first time on *P. patula* and *P. maximinoi*, two species that are important components of the commercial forestry industry in various southern hemisphere countries. The sexual state of *L. pharomachri* was also discovered for the first time.

L. pharomachri was first described in a study considering the presence of cryptic *Lecanosticta* spp. collected in Guatemala, Honduras, Nicaragua, and Mexico on different native *Pinus* spp. (van der Nest et al. 2019b). Its description emerged from analyses of DNA sequence data, and the species was characterized based on asexual structures of the fungus grown in culture. The discovery of *L. pharomachri* on pine needles in Colombia provided the first opportunity to examine asexual structures from naturally infected tissues, and structures of the sexual state could also be characterized for the first time. This made it possible to substantially improve and formally emend the description of the pathogen. The similarity between the morphological features of *L. pharomachri* and those of *L. acicola* (Table 2) and other closely related species from Central America (European and Mediterranean Plant Protection Organization 2015; Quaedvlieg et al. 2012; Wolf and Barbour 1941) emphasizes the importance of using DNA sequence data for the identification of *Lecanosticta* spp. (van der Nest et al. 2019b).

An interesting outcome of this study, which included a large collection of isolates from a wide diversity of locations and different hosts, was that it resolved as the single species *L. pharomachri*. Although the presence of *L. acicola* has been confirmed in Colombia (Janoušek et al. 2016), this species was not isolated in any of the samples collected over a wide area of Colombia. This could imply that *L. acicola* is less pathogenic and not able to compete with *L. pharomachri* on the *Pinus* spp. most widely propagated in Colombia. A more probable hypothesis is that the serious outbreaks of BSNB in the past, and considered by Evans (1984), were because *P. radiata* was commonly planted at that time and was highly susceptible to *L. acicola*. *L. acicola* has previously been reported on *P. patula* from Colombia and Mexico, and *P. maximinoi* and *P. tecunumanii* from Honduras and Nicaragua (Evans 1984; Gibson 1980). However, those reports relied on morphological observations and need to be verified using DNA sequence comparisons because they could easily represent other species of *Lecanosticta.*

L. pharomachri appears to have become established relatively rapidly over a large area where *Pinus* spp. are being propagated in Colombia. This view is based on the fact that symptoms of the disease now known to be caused by this pathogen were virtually unknown before 2016 (Wingfield and Rodas, unpublished data). It is unlikely that needle infections by *L. pharomachri* have been confused with DNB, which first emerged as a serious problem in 2008 (Rodas et al. 2016). This view is supported by the fact that the pine species severely damaged by *L. pharomachri* in this study are either not susceptible (*P. patula* and *P. maximinoi*) or only mildly susceptible (*P. kesiya* Royle ex Gordon) to DNB. This would imply that needle disease outbreaks caused by *L. pharomachri* represent an important and emerging problem in Colombia, especially on the widely planted *P. patula*, and that it is likely to become increasingly relevant in the future.

The DNA sequence data generated in this study showed that the population of *L. pharomachri* is relatively diverse based on the number of *TEF*1 and *RPB*2 haplotypes found. This would suggest that numerous haplotypes of *L. pharomachri* have been introduced into Colombia. Alternatively, different haplotypes could have originated as a product of recombination by sexual reproduction, explaining why the *TEF*1 haplotypes of the pathogen in Colombia are different from those known from areas where the pathogen has been found previously. Ascomata of *L. pharomachri* were very rarely encountered in this study, suggesting that sexual reproduction is unlikely to account for the genetic variation between isolates from Colombia.

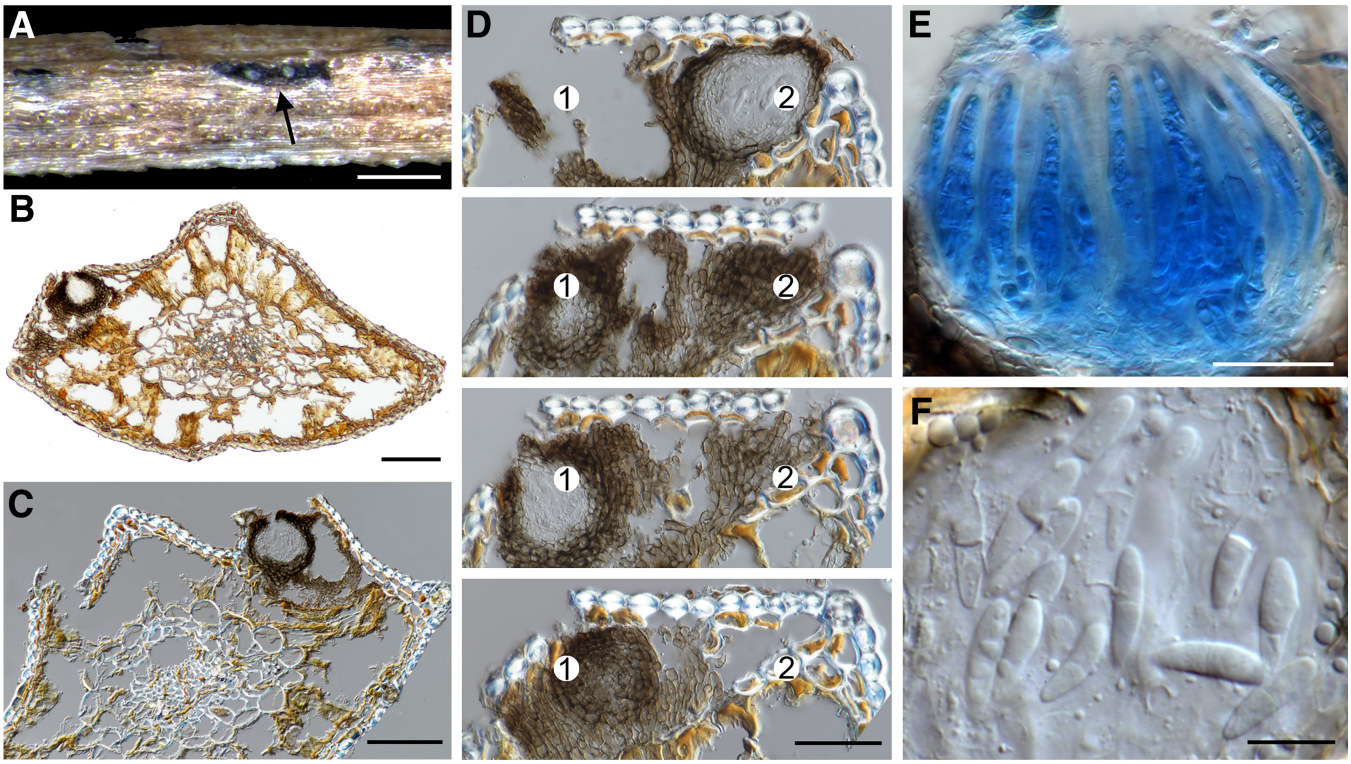

Fig. 3. Micrographs of the sexual state of *Lecanosticta pharomachri* on *Pinus patula*. **A,** Ascostroma (with two locules) in the needle with top part removed (arrow). **B and C,** Cross section of needle with ascostroma. **D,** Serial section along the length of the needle of a biloculate (locule 1 and 2) ascostroma. **E,** Asci stained with cotton blue. **F,** Ascospores. Scale bars: A = 250 µm; B and C = 100 µm; D = 50 µm; E = 25 µm; F = 10 µm.

Most of the *Pinus* spp. propagated in Colombia are native in areas where *L. pharomachri* has previously been recorded. Therefore, it seems more plausible that multiple introductions of the pathogen have occurred with plant material used to establish plantations in the country. In this regard, it is well known that various leaf and shoot pathogens of tree species propagated as non-natives in countries practicing plantation forestry have been introduced by anthropogenic activities (Barnes et al. 2014; Cleary et al. 2019b; Janoušek et al. 2016).

L. acicola is emerging as one of the most important pathogens of *Pinus* spp., particularly in northern hemisphere countries. Although most likely native to Mesoamerica (Janoušek et al. 2016; van der Nest et al. 2019b), it has been accidentally introduced into various countries of Europe where it is causing serious disease problems (Cleary et al. 2019a; Janoušek et al. 2016; Mesanza et al. 2021b; Mullett et al. 2018). *L. pharomachri* is a very similar pathogen, and results of this study show that it has a wider host range than was previously known. Persistent sexual outcrossing in the populations of *L. pharomachri* could increase genetic diversity, and with the high rate of asexual reproduction, would increase the probability of its further spread to new areas. Therefore, it will be important to monitor the spread of *L. pharomachri* as a potential threat to plantations in the southern hemisphere where Mesoamerican *Pinus* spp., such as those sampled in this study, are becoming increasingly important for commercial plantation establishment.

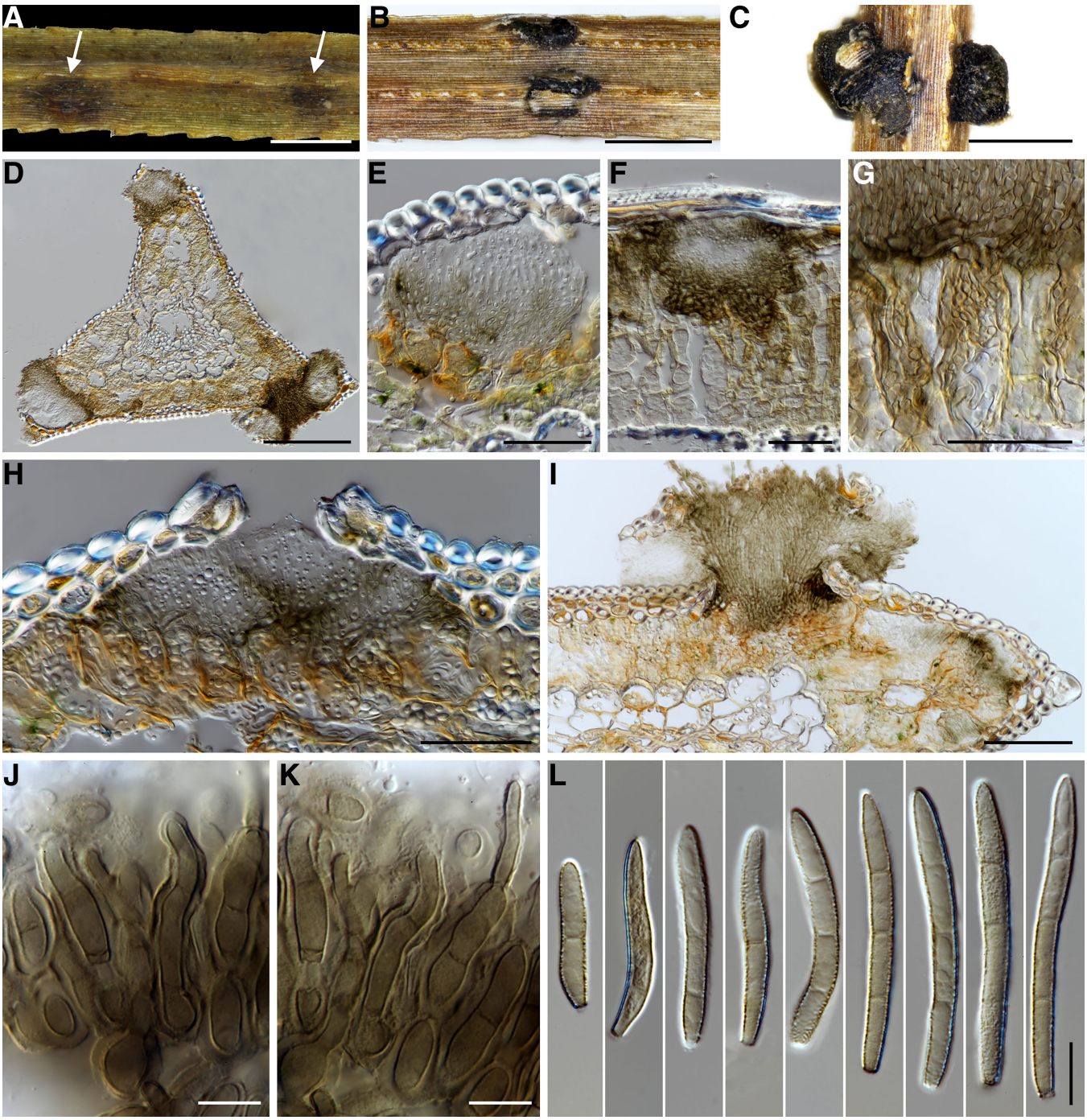

Fig. 4. Micrographs of the asexual state of *Lecanosticta pharomachri* on *Pinus maximinoi*. **A,** Needle showing the evidence of young innate conidiomata in brown patches (arrows). **B,** Conidiomata bursting through epidermis. **C,** Pulvinate conidiomata. **D,** Cross section of needle with three mature conidiomata. **E and F,** Innate conidiomata. **G,** Stromatic tissues (upper) and vegetative hyphae growing in mesophyll cells. **H,** Conidioma opening the epidermis. **I,** Fully erumpent conidioma. **J and K,** Conidiogenous cells. **L,** Conidia. Scale bars: A to C = 500 µm; D = 250 µm; E to H = 50 µm; I = 100 µm; J to L = 10 µm.

Table 2. Morphological features used to distinguish *Lecanosticta acicola* and *L. pharomachri*

Feature	*L. acicola*	*L. pharomachri*
Needle symptoms	Initially yellow to gray-green spots at the site of infection, turns brown over time, often surrounded by a chlorotic halo, needles die from the apex to the base and are eventually cast	Initially gray-brown spot at the site of infection, no chlorotic halo or brown discoloration, erumpent conidiomata develop, and needles occasionally die from the apex to the base
Asexual state		
Conidiomata	Pseudopycnidial to acervular, subepidermal becoming erumpent when mature, 200–800 × 150–200 µm, thick-walled pseudoparenchymatous cells; hyphae intracellular, confined to mesophyll cells, not beyond the endodermis, large, dark, short-celled hyphae	Acervular, subepidermal, dehiscing epidermis becoming erumpent when mature, pulvinate, 63–275 × 82–424 µm (171 ± 58.7 × 223 ± 89.7 µm), prosenchymatous or pseudoparenchymatous, composed of subhyaline, thick-walled cells; vegetative hyphae present beyond stromatic structure toward mesophyll, not beyond endodermis, thick-walled, pale brown to brown
Conidiophores	Subcylindrical, densely aggregated, dark brown, verruculose, unbranched or branched at base, septate, (18–) 20–60 × (2.5–) 4–6 µm	Cylindrical, pale brown, septate, surrounded by mucilaginous substance, 26–52 × 3–6 µm (35.3 ± 7.33 × 4.8 ± 0.61 µm)
Conidiogenous cells	Holoblastic, terminal, integrated, subcylindrical, pale brown, verruculose	Holoblastic, integrate, cylindrical, hyaline
Conidia	Subcylindrical, straight to curved, with obtusely rounded apex, base truncate, subhyaline to light brown or olive, guttulate, echinulate to verrucose, 1–5-septate, base 2.5–3.5 µm diam, (17–) 30–45 (–55) × (3–) 4 (–4.5) µm	Fusiform to cylindrical, straight, or slightly curved, tapering toward apex and base, base truncated, subhyaline to pale brown, verruculose, 1–3-septate, 28–49 × 3–5 µm (39.5 ± 4.38 × 3.8 ± 0.38 µm)
Sexual state		
Ascostromata	Irregularly dispersed in dead needle tissue, subepidermal, becoming erumpent and split epidermis when mature, ostiolate; pseudoparenchymatous, thick-walled brown cells	Immersed in host tissues, subepidermal or subhypodermal, arranged longitudinally along length of needle, bursting through epidermis at maturity, opening via an ostiole, ostiole reaching surface; stromatic tissues pseudoparenchymatous, composed of thick-walled, brown cells
Locules	Uni- to multiloculate, globose to flask-shaped, periphysate, 50–70 × 50–80 µm	Multiloculate, subglobose to obpyriform, 82–110 × 71–94 µm (95.5 ± 9.0 × 84.1 ± 7.4 µm)
Asci	Bitunicate, saccate to cylindrical, 8 spores, 25–55 × 6.5–10.5 µm	Bitunicate, narrowly cylindrical to clavate, 8-spored, 45–52 × 5–8 µm (47.8 ± 2.34 × 6.9 ± 0.82 µm)
Ascospores	Hyaline to light brown, smooth, elliptical, unequally 2-celled with larger upper cell, 1-septate, 7.5–19 × 2–3.5 µm (15.1 ± 4.8 µm × 3.3 ± 0.31 µm)	Hyaline, smooth, fusoid, 2-celled, straight, or slightly curved, septum median to supra-median, slightly constricted, 10–15 × 3–4 µm (13.03 ± 1.26 × 3.25 ± 0.31 µm)
Culture morphology (2% MEA)	Stromatic, green-olive to olive-gray surface, produces conidia in a dark olive-colored slime, yellow diffuse in agar	Stromatic, with fluffy aerial mycelium at midpoint, cinnamon to apricot edges, produce conidia in greenish olivaceous honey mass, yellow diffuse in agar

Acknowledgments

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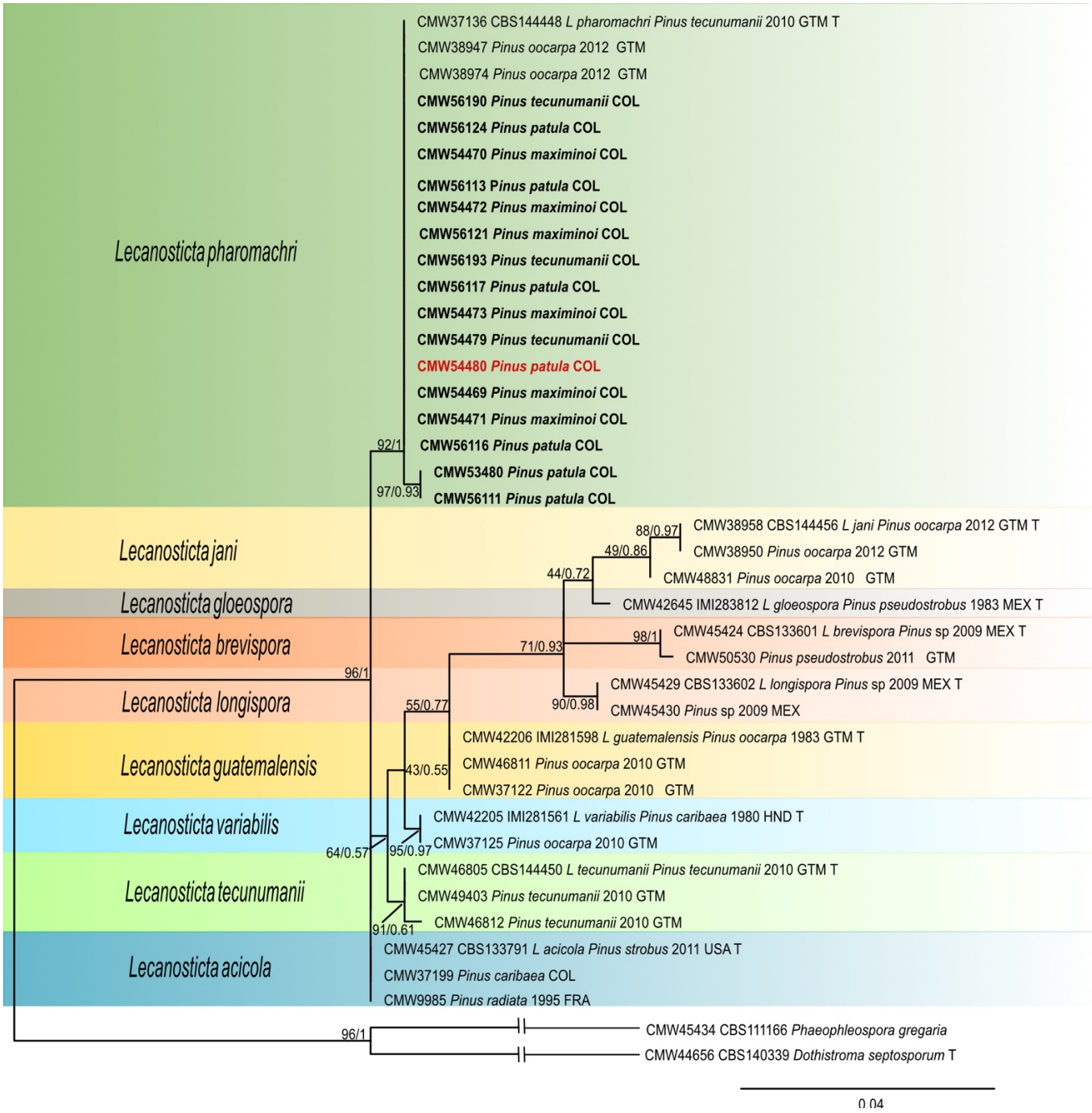

Fig. S1. Maximum-likelihood tree based on the ITS regions for all *Lecanosticta* species. Isolates collected in this study, indicated in bold, fall within the *Lecanosticta pharomachri* clade. The sequence from the sexual state of *L. pharomachri* is indicated in red. Ex-type species are indicated with a "T" and *Dothistroma septosporum* and *Phaeophleospora gregaria* were used as the outgroup taxa. Numbers at nodes indicate the bootstrap support (n=1000) and posterior probabilities from Bayesian inference, respectively.

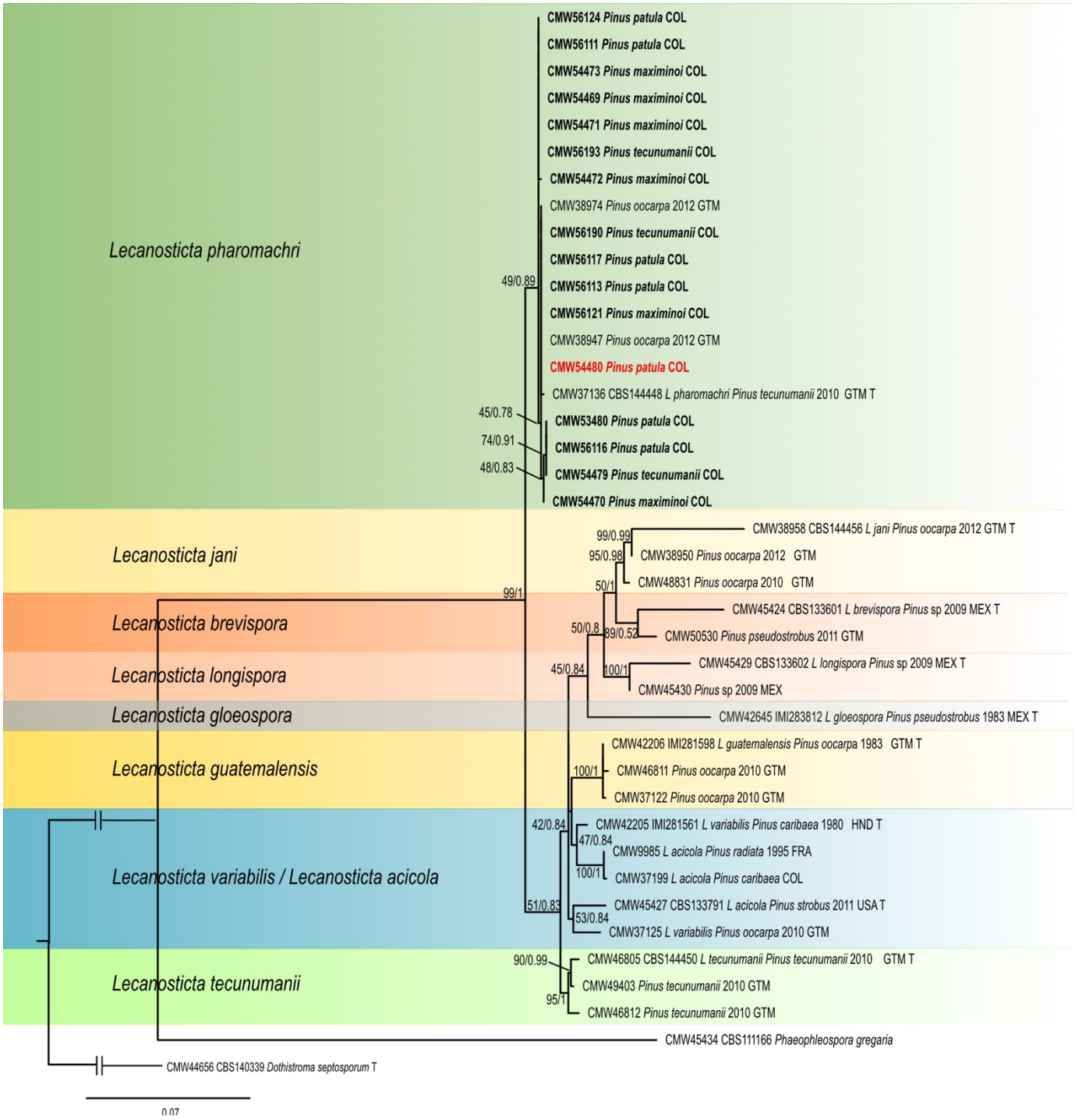

Fig. S2. Maximum-likelihood tree based on the *RPB*2 regions for all *Lecanosticta* species. Isolates collected in this study, indicated in bold, fall within the *Lecanosticta pharomachri* clade. The sequence from the sexual state of *L. pharomachri* is indicated in red. Ex-type species are indicated with a "T" and *Dothistroma septosporum* and *Phaeophleospora gregaria* were used as the outgroup taxa. Numbers at nodes indicate the bootstrap support (*n*=1000) and posterior probabilities from Bayesian inference, respectively.

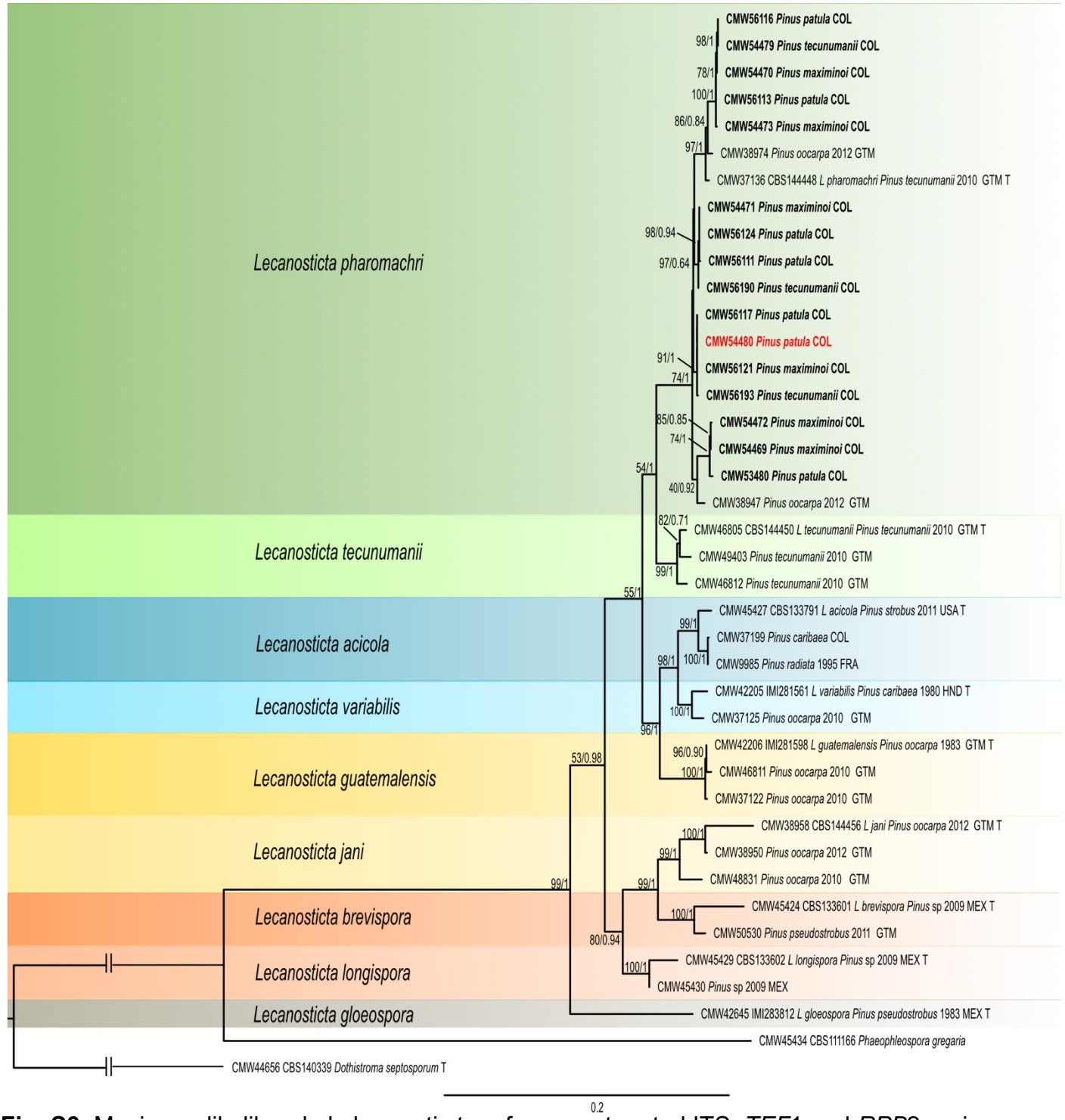

Fig. S3. Maximum-likelihood phylogenetic tree for concatenated ITS, *TEF*1 and *RPB*2 regions for all *Lecanosticta* species. Isolates collected in this study, indicated in bold, fall within the *Lecanosticta pharomachri* clade. The sequence from the sexual state of *L. pharomachri* is indicated in red. Ex-type specimens are indicated with a "T" and *Dothistroma septosporum* and *Phaeophleospora gregaria* were used as the outgroup taxa. Numbers at nodes indicate the bootstrap support (*n*=1000) and posterior probabilities from Bayesian inference, respectively.

CHAPTER 3

The diversity and distribution of *Lophodermium* species on non-native *Pinus* species in the Southern Hemisphere

ABSTRACT

Lophodermium spp. are amongst the most commonly isolated endophytic fungi on the needles and cones of *Pinus* spp. Of the 38 species reported on *Pinus* spp., only *Lophodermium seditiosum* is considered to be a major pathogen. *Pinus* spp. have been widely established as non-natives in Southern Hemisphere countries and several *Lophodermium* spp. have been reported on the needles of these trees. However, the majority of these reports are based on morphology alone and it is not known whether any new incursions have occurred. Therefore, the aim of this study was to determine the biogeography of *Lophodermium* spp. throughout the Southern Hemisphere by obtaining and identifying isolates from non-native *Pinus* spp. planted in Australia, Chile, Colombia, New Zealand and South Africa. More than 100 isolates were sourced from culture collections or isolated directly from symptomatic needles. A multi-locus (ITS, *ACT* and *TEF*1) phylogenetic approach was used to delineate the species and characteristic morphological features were evaluated against the resulting phylogeny. The phylogenetic analyses revealed the presence of five *Lophodermium* taxa previously reported in the Southern Hemisphere. A species belonging to the *L. conigenum-australe* complex was found in all countries, except for Chile. *Lophodermium indianum* and *L. molitoris* were found only in Colombian and New Zealand collections, respectively. Two distinct lineages of *L. pinastri* emerged of which one included isolates from Chile and New Zealand and the other from Australia. Morphological descriptions were mostly consistent with the descriptions of the species, but a high level of variation was observed for several of the characteristics examined. New hosts were recorded for a species from the *L. conigenum-australe* complex and *L. indianum*. The results from the study support the fact that various cryptic *Lophodermium* spp. occur on *Pinus* spp. and suggests that several independent introductions of these fungi have occurred into Southern Hemisphere countries, most likely with seed and plant importations.

1. INTRODUCTION

Endophytic fungi of plants are mainly horizontally transferred and the community composition is highly dependent on the host and geographic location (Christian et al., 2016). In conifers, where needles are retained for long periods of time, endophytic communities are found to be relatively diverse (Sieber, 2007). Endophytic studies of the fungi infecting needles on *Pinus* spp. native to the Northern Hemisphere have found members of *Rhytismataceae* to be dominant (Carroll and Carroll, 1978; Hata and Futai, 1996; Ortiz-García et al., 2003; Ganley et al., 2004). This includes species of *Lophodermium* Chevall., for which approximately 38 species have been described from needles and cones (Salas-Lizana and Oono, 2018b). The majority of the *Lophodermium* spp. are recognized as endophytes of healthy needles that typically sporulate when the needles die (Minter and Millar, 1980; Minter, 1981). *Lophodermium seditiosum* Minter*,* Staley & Millar is the only species considered a primary pathogen, causing needle cast which may result in a reduction in height and diameter and occasionally seedling death in nurseries (Minter and Millar, 1980; Staley and Nicholls, 1989; Jansons et al., 2020).

The majority of *Lophodermium* spp. have been described based on the internal and external morphological features of the ascomata on the needles (Minter, 1981). The internal features include the placement of the ascoma relative to the epidermis and hypodermis of the needles, the number and arrangement of epidermal cells displaced, and the presence and pigmentation of the clypeus. External features include the presence or absence of zone lines on the needle surface, the colour and shape of the ascomata, and their position relative to the stomata (Darker, 1967; Minter, 1981). These features are known to be highly variable, and most likely depend on the needle anatomies of the trees the *Lophodermium* sp. is colonizing (Minter, 1981; Johnston et al., 2003; Ortiz-García et al., 2003).

The first comprehensive phylogenetic study on *Lophodermium* spp. utilized sequences for the internal transcribed spacer rDNA (ITS) region and this revealed inconsistencies in the species identity based on morphological characters (Ortiz-García et al., 2003). DNA sequence analyses on isolates identified as *Lophodermium pinastri* (Schrad.) Chevall*.* showed substantial sequence diversity in the ITS region for isolates from North America, Europe and New Zealand, strongly suggesting the occurrence of at least five cryptic taxa (Johnston et al., 2003; Ortiz-García et al., 2003; Reignoux et al., 2014). A morphological study by Minter (1981) highlighted the

similarities in the ascomata of *Lophodermium conigenum* (Brunaud) Hilitzer and *Lophodermium australe* Dearn. Subsequently, phylogenetic analyses using the ITS region suggested that these species are conspecific (Prihatini et al., 2016; Salas-Lizana and Oono, 2018a).

Several studies have recently been performed on the endophytic communities of native and planted *Pinus* spp. in the Northern Hemisphere and have recovered, based on molecular data, previously unidentified species of *Lophodermium* (Sokolski et al., 2004; Hou et al., 2009; Koukol et al., 2015; Tanney and Seifert, 2017; Salas-Lizana and Oono, 2018b). Several of these studies adopted the partial actin (*ACT*) gene region as a secondary marker, which still did not provide better resolution than the ITS region for the *Lophodermium* spp. tested (Koukol et al., 2015; Tanney and Seifert, 2017; Salas-Lizana and Oono, 2018b). The translation elongation factor 1-α (*TEF*1) gene was identified as a promising phylogenetic marker and previous studies suggested that it could be useful in phylogenetic studies of the *Rhytismatales* in the future (Stielow et al., 2015; Tanney and Seifert, 2017).

Plantations of *Pinus* spp. were established in various Southern Hemisphere countries late in the 19th century. Concurrent with this development, numerous pathogens appeared on these trees including those that were inadvertently introduced along with planting material (Gibson, 1979; Wingfield, 1999; Burgess and Wingfield, 2001). One of the earliest reports of *Lophodermium* spp. in the Southern Hemisphere was of *Hysterium pinastri* (now *L. pinastri*) causing tip dieback in *Pinus radiata* D. Don and *Pinus pinea* L. in South Africa (Zahn and Neethling, 1929; Lundquist, 1987). Since then, several species of *Lophodermium* have been reported on *Pinus* spp. in various Southern Hemisphere countries.

Most reports of *Lophodermium* spp. on non-native *Pinus* spp. in the Southern Hemisphere have relied solely on morphological characteristics for identification. Thus, species such as the purported pathogen, *L. seditiosum*, has been reported from both Chile and South Africa (Roux and Lundquist, 1984; Butin and Peredo, 1986) however, there has never been any evidence of disease caused by this fungus. This calls into question the reliability of those, and most likely other, reports of these fungi. Identifications based on DNA sequence data has only been performed for a small number of isolates from *Pinus* spp. specifically in Australia and New Zealand. These studies have recorded the presence of *L. conigenum* and *L. pinastri* in both countries

and *L. molitoris* Minter in New Zealand (Johnston et al., 2003; Ortiz-García et al., 2003; Prihatini et al., 2015).

The aim of this study was to confirm the identity, historically based on morphology, of *Lophodermium* spp. collected from *Pinus* spp. in Southern Hemisphere countries using DNA sequence data. Due to the availability of cultures or samples, and well-established commercial *Pinus* plantations, we focussed specifically on Australia, Chile, Colombia, New Zealand and South Africa. The objectives were to: 1) obtain a large collection of *Lophodermium* samples from non-native *Pinus* spp. grown in these five countries, 2) isolate and confirm the identity of the fungal species using DNA sequence and phylogenetic analyses of the ITS and *ACT* regions, 3) determine the suitability of the translation elongation factor 1-α gene region (*TEF*1) in resolving the identity of the *Lophodermium* spp. isolated and 4) investigate and compare the external and internal morphological features of the ascomata from different *Pinus* spp. and countries, and the culture morphology, with the resulting phylogeny to determine their usefulness in delimiting the different species. A broader objective was to consider the results from a biogeographic perspective and to identify the possible origins and patterns of distribution of *Lophodermium* spp. in Southern Hemisphere countries.

2. MATERIALS AND METHODS

2.1. Sample collection and isolations

Isolates of *Lophodermium* spp. were obtained either directly from infected needles collected in Australia, Chile, Colombia and South Africa or from isolates preserved in New Zealand and South African culture collections. Although Colombia is not strictly in the Southern Hemisphere, it was included in this study due to the non-native nature of plantation grown *Pinus* spp. in the country that has a similar history as the other countries examined in this study. Needles bearing ascomata resembling *Lophodermium* spp. were collected from trees between February 2020 and March 2021. The needles were placed in labelled paper bags inside large plastic zip lock bags and stored at 4ºC before shipping and processing at the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa. Needles were inspected under a light microscope and a subset of these with characteristic ascomata (Minter, 1981) were chosen from different sites and hosts from each country (Table 1). Needles were surface sterilized by spraying with 70% ethanol, wiped down using autoclaved, distilled water and paper towels and placed in moist

chambers for 4 hours or overnight. Ascomata from hydrated needles were then excised using a clean scalpel blade and macerated on the surface of 2% malt extract agar (MEA, 20 g/L malt extract, 20 g/L agar; Biolab). Cultures were incubated at 20 - 25ºC for 24 - 48 hours, after which single germinating spores or hyphal tips were sub-cultured onto 2% MEA to obtain pure cultures.

Herbarium specimens of *Lophodermium* spp. were obtained from the National Forestry Mycological Herbarium (NZFRI-M), Scion, New Zealand. Cultures from New Zealand were supplied by the National Forest Culture Collection (NZFS), Scion, New Zealand. Cultures from Colombia, Guatemala and South Africa were retrieved from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. All isolates were plated onto 2% MEA and after one week those from culture collections and isolated in this study were grouped into morphotypes of tentative *Lophodermium* spp. (Minter, 1981). All isolates used in this study have been preserved in the CMW culture collection.

2.2. DNA extraction and PCR amplifications

One-week-old cultures were used in DNA extraction by macerating fungal mycelium with a sterile toothpick and using Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. PCR amplification of the isolates were performed for the internal transcribed spacer rDNA (ITS) region, amplified using the primers ITS1 and ITS4 (White et al., 1990), partial actin (*ACT*) gene region using ACT-512F and ACT-783R (Carbone and Kohn, 1999) and the translation elongation factor 1-α (*TEF*1) gene region using the primers EF-728F and EF-986R (Carbone and Kohn, 1999). PCR amplifications were performed in 13 µL reactions containing 2.5 µL 5 x MyTaq buffer (Bioline, London, UK), 0.25 µL MyTaq DNA polymerases (Bioline), 1 µL DNA template, 0.5 µL of each primer (10 mM), and 8.25 µL sterile SABAX water. PCR conditions for all reactions were as Tanney and Seifert (2017) on an Applied Biosystems Veriti© 96 well Thermal cycler (Thermo Fisher Scientific, MA, USA). Amplification of all PCR products was confirmed by staining 2 µL product with 1 µL GelRed™ Nucleic Acid Gel Stain (Biotium, CA, USA) and separating these on a 2% agarose gel at 90 V for 30 min, after which they were visualized under UV light.

For herbarium specimens, DNA was extracted directly from single ascoma, which were crushed in 30 µL TE Buffer (10mM Tris, 1mM EDTA) using a sterilized

toothpick and heated at 95ºC for 15 min followed by 80ºC for 2 min. The primers ITS1-F (Gardes and Bruns, 1993) and ITS4 were used to amplify the ITS region and amplification was confirmed as above.

2.3. Sequencing and phylogenetic analyses

PCR products were cleaned in a 6.7% G-50 Sephadex solution (MilliporeSigma, MA, USA) using Centri-sep columns (Princeton Separations, NJ, USA) following the manufacturer's protocols. Sequencing reactions were conducted for both the forward and reverse strands in 12 µL reaction volumes. The reactions contained 3 µL PCR product, 1 µL BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Thermo Fisher Scientific, MA, USA), 2 µL sequencing buffer, 1 µL of either the forward or reverse primer and 5 µL sterile SABAX water and sequenced on the ABI PRISM™ 3500xl Auto-sequencer (Applied Biosystems, Thermo Fisher Scientific, MA, USA) at the sequencing facility of the University of Pretoria. The forward and reverse sequencing reads were imported into CLC Bio Main Workbench v8 (CLC Bio, https://www.qiagenbioinformatics.com/products/clc-main-workbench/) to construct contigs and consensus sequences. The ITS sequences were used for a BLASTn analysis against the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov) to confirm that they were *Lophodermium* spp.

Previously deposited ITS sequences for isolates of *Lophodermium* collected in Colombia, New Zealand and Guatemala were retrieved from GenBank and analysed together with the sequences generated in this study (Supplementary Table S1). Sequences for ITS and *ACT* with similar species identity were aligned in CLC Main Workbench and alignments were visually inspected to confirm nucleotide differences and to determine the number of unique haplotypes for each gene region.

A representative for each unique ITS and *ACT* haplotype (Supplementary Table S1) was chosen for collections from all six countries and used for downstream phylogenetic analysis (Table 1). Subsequently, the *TEF*1 gene region was amplified for all of the representatives chosen and phylogenetic analyses were performed to evaluate whether sequences for this region could discern *Lophodermium* spp. ITS, *ACT* and *TEF*1 sequences for closely related taxa from different countries were retrieved from public databases (Supplementary Table S2), with a focus on the species known to be associated with *Pinus* spp. These sequences were added to the sequences from the present study to compile datasets for phylogenetic analyses.

Datasets for the ITS, *ACT* and *TEF*1 were aligned using the MAFFT v7 online resource (https://mafft.cbrc.jp/alignment/server/), with default settings, and visually inspected in BioEdit Sequence Alignment Editor v7.0.9.0 (Hall, 1999). IQ-Tree Web server (http://iqtree.cibiv.univie.ac.at/) was used for maximum likelihood (ML) analyses and to determine the number of parsimony-informative sites. The best ranked model for each dataset was determined according to the Bayesian information criterion (BIC) by ModelFinder (Kalyaanamoorthy et al., 2017) and ML trees were constructed with confidence levels estimated using 1000 bootstrap replicates.

Bayesian inference of phylogeny was performed for each of the datasets using Mr Bayes v3.2.7a (Ronquist et al., 2012) following the model as stipulated by ModelFinder. For the analyses, two Markov Chain Monte Carlo (MCMC) chains were set to run for 3 million generations, with sampling every 100 generations, and the temperature of the chain set to 0.01. Stationarity was visualized in Tracer v1.7.2 and the first 25% of generations were discarded as burn-in. Based on previous studies, *Colpoma quercinum* and *Tryblidiopsis pinastri* were used as the outgroup taxa for the ITS phylogeny (Koukol et al., 2015; Bartnik et al., 2021) and *Hymenoscyphus epiphyllus* for the *ACT* phylogeny (Koukol et al., 2015; Li et al., 2016; Tanney and Seifert, 2017). Due to the limited sequence data available for the *TEF*1 region for the *Rhytismatales*, *Strasseria geniculata* was used as the outgroup taxon according to its placement relative to *Lophodermium* spp. in previous studies (Prihatini et al., 2014). Phylogenetic trees were visualized in FigTree v1.4.4 and edited in Affinity Designer v1.10.4.

2.4. Morphological observations

Morphological characteristics of the ascomata were studied using structures taken directly from the needles for representative specimens of the *Lophodermium* spp. based on the clades observed in the phylogenetic analyses (Table 1). This included specimens from each of the sampled countries and different *Pinus* spp. The colour, shape and size of the ascomata and the presence and colour of the clypeus and zone lines on the needle surface were observed using a ZEISS SteREO Discovery V12 stereomicroscope and photographed under a ZEISS Camera AxioCam ICc5 (Carl Zeiss AG, Oberkochen Germany). To observe the position of the ascoma in the host tissue, the arrangement of the cells and the structure of the basal wall, dry needles were softened by placing them between moist paper towels for two hours. Needles were then cut into small fragments containing a single mature ascoma and were

subsequently embedded in tissue freezing medium (Leica Biosystems, Buffalo Grove, IL). Sections (50 µm thickness) were cut transversally across the needle using a freezing microtome (Leica Biosystems, Buffalo Grove, IL). The sections, asci and ascospores were mounted in 85% lactic acid and observed using a Nikon H550L microscope (Nikon, Yokohama, Japan) and a camera (Nikon DS-Ri2).

Colony characteristics and pigment formation were observed for representative isolates sub-cultured onto 2% MEA and filtered ground pine needle agar of *Pinus radiata* (FGPNA) (Luchi et al., 2007), and incubated in the dark at 25ºC for 8 - 10 weeks. Conidia produced in culture were mounted in water, later replaced with 85% lactic acid, and 20 measurements were taken for each of the isolates. All measurements for the length and width were described as minimum to maximum (average).

3. RESULTS

3.1. Sample collection and isolations

A total of 107 isolates tentatively identified as *Lophodermium* spp. were obtained either from isolations made from ascomata in this study or previously preserved cultures requested from culture collections (Table 1). These included 15 isolates from Australia, 12 from Chile, 18 from New Zealand, 29 from South Africa, 27 from Colombia and eight from Guatemala.

3.2. Sequencing and sequence analyses

The ITS region was successfully amplified and sequenced for 94 isolates originating in the six countries considered (excluding the sequences for 13 isolates retrieved from GenBank). Two ITS amplicons were successfully generated from a direct PCR of two individual ascoma from the herbarium specimens (NZFRI-M3975, NZFRI-M4654, NZFRI-M2850) from New Zealand. The relative amplicon length for all ITS sequences generated was 542 bp. BLASTn analyses against the GenBank database using sequences for the ITS region tentatively identified most isolates as a species of *Lophodermium*. The 96 ITS sequences generated in this study were added to the sequences retrieved from GenBank and alignments revealed a total of 42 unique ITS haplotypes (Supplementary Table S1). A subset of 67 ITS sequences representing the 42 unique haplotypes for isolates from the six sampled countries and those from

different *Pinus* spp. were used for the phylogenetic analysis. All sequences generated in this study for the ITS region were deposited in GenBank (Supplementary Table S1).

The *ACT* region was successfully amplified for 109 isolates (Supplementary Table S1). The average fragment length of the sequences was 267bp and a total of 47 unique *ACT* haplotypes were observed (Supplementary Table S1). A subset of 66 *ACT* sequences representing the unique *ACT* haplotypes were used for the phylogenetic analysis.

The *TEF*1 gene region was successfully amplified for 59 of the representative isolates based on the ITS and *ACT* haplotypes obtained and the average fragment length was 214bp. Sequence alignments revealed 32 unique *TEF*1 haplotypes and all 59 sequences were used in the phylogenetic analyses (Supplementary Table S1).

3.3. Phylogenetic analyses

High levels of nucleotide variability were observed in the sequences across all three gene regions for the *Lophodermium* spp. Sequences for ex-type specimens for species in the *Rhytismatales* were clearly underrepresented and a lack of sequences available for the *ACT* and *TEF*1 regions precluded a combined analyses in this study. Therefore, individual datasets were consequently used. The ITS dataset consisted of 124 sequences with 570 aligned characters and 156 parsimony-informative sites. The *ACT* dataset consisted of 84 sequences with 392 aligned characters and 137 parsimony-informative sites. The *TEF*1 dataset consisted of 67 sequences with 263 aligned characters and 157 parsimony-informative sites. The best fit substitution model according to the Bayesian information criterion (BIC): TIM2e+I+G4 for ITS, TIM2e+G4 for *ACT* and HKY+F+G4 for *TEF*1. The ML and BI analyses provided similar tree topologies for all three regions and the ML trees were selected for presentation (Fig. 1, 2 and 3).

Phylogenetic analyses for the ITS (Fig. 1), *ACT* (Fig. 2) and *TEF*1 (Fig. 3) regions showed that there were five strongly supported clades of *Lophodermium* present on non-native *Pinus* spp. planted in the Southern Hemisphere. These clades closely align to *Lophodermium* spp. previously characterized for these gene regions. No new reports of *Lophodermium* spp. were found for the countries considered and apart from the *L. conigenum-australe* complex, there is little overlap in the species present in the different countries from the Southern Hemisphere. The phylogenetic

clades in Fig. 2 and Fig. 3 are labelled according to the clades assigned in Fig. 1 for the ITS analysis.

Clade 1. *Lophodermium conigenum-australe* complex

A majority of the sequences fell within clade 1, containing isolates collected from Australia, Colombia, New Zealand and South Africa from ten different hosts. The ITS analyses revealed nineteen unique ITS haplotypes (Supplementary Table S1) that grouped with sequences for both *L. australe* from the United States of America (USA) and Mexico and *L. conigenum* from China, Australia and New Zealand with a bootstrap of 85% (Fig. 1). The *ACT* analyses revealed 20 unique *ACT* haplotypes (Supplementary Table S1), where the majority of the isolates group with two sequences available for *L. australe* from the USA with a bootstrap support of 100% (Fig. 2). However, three sequences representing three different haplotypes from Australia and South Africa formed a sister group to these isolates with a bootstrap support of 97% (Fig. 3). Therefore, until more sequence data becomes available to utilise a combined gene tree, isolates that grouped in clade 1 from the four countries were identified as belonging to the *L. conigenum-australe* complex. This clade was also well supported in the *TEF*1 analysis, revealing 11 unique haplotypes (Supplementary Table S1) that grouped with a bootstrap support of 89% (Fig. 3).

Clade 2. *Lophodermium indianum*

Clade 2 accommodated only sequences from Colombia and Guatemala collected from Mesoamerican *Pinus* spp. and their hybrids (*P. maximinoi, P. tecunumanii, P. patula, P. patula x P. tecunumanii*). This clade revealed 10 distinct haplotypes (Supplementary Table S1) that resolved into two subclades in all three regions analysed with a bootstrap support > 75%. Subclade 1 contained the isolates from Colombia and the isolates from Guatemala grouped into subclade 2. Subclade 1 contained seven ITS haplotypes and grouped with sequences previously identified as *L. indianum* S.Singh & D.W.Minter from Mexico with a bootstrap support of 85% (Fig. 1). Subclade 2 grouped with a bootstrap support of 99% (Fig. 1) and only contained isolates from Guatemala. The *ACT* analysis revealed 11 unique haplotypes (Supplementary Table S1). However, the topology of the isolates from subclade 1 changed depending on the gene regions analysed. For the *ACT* analysis, the isolates from Colombia formed a polytomy (Fig. 2). Furthermore, in the *TEF*1 analysis, several

Colombian isolates were paraphyletic to the rest of the Colombian isolates (Fig. 3). Therefore, all isolates from clade 2 are recognized as *L. indianum* until additional sequence data becomes available.

One haplotype from Chile on *P. radiata* (CMW58479) grouped close to, but separate from, clade 2 in all the gene regions analysed (Fig. 1, 2, 3). This haplotype, representing four isolates, was tentatively identified as *Meloderma desmazierii* (Duby) Darker with a percentage identity of 100% based on the NCBI BLASTn analysis (Accession No. KY485137). In the ITS analysis, this sequence groups with an isolate of *M. desmazierii* from Canada. However, no additional sequence data were available for this species for the other gene regions and in the *ACT* and *TEF*1 analyses this haplotype groups on its own.

Clade 3. *Lophodermium pinastri* A

The ITS, *ACT* and *TEF*1 analyses grouped the isolates from clade 3 into two separate subclades. Subclade 1 contained isolates from New Zealand collected from five different *Pinus* spp. For the ITS analyses, these isolates revealed a single ITS haplotype (Supplementary Table S1) that grouped directly with sequences of *L. pinastri* previously identified in New Zealand and which are closely related to isolates for *L. pinastri* from East Asia (Fig. 1). The *ACT* (Fig. 2) and *TEF*1 (Fig. 3) analyses revealed two different haplotypes for these isolates (Supplementary Table S1) which group together with a high statistical support with isolates of *L. pinastri* from Scotland and New Zealand. Subclade 2 contained isolates from Chile, all collected from *P. radiata,* which showed high nucleotide variation resulting in six and seven haplotypes for the ITS and *ACT* analyses, respectively (Supplementary Table S1). In the ITS and *ACT* analyses, these isolates grouped with sequences of *L. pinastri* from Scotland (Fig. 1, 2). In the *TEF*1 analysis, the sequences from Chile resulted in five haplotypes (Supplementary Table 1) that formed two lineages (Fig. 3). Therefore, until more sequence data becomes available to resolve the subclades as potentially different species, all isolates falling in this clade are identified as *L. pinastri* A.

Clade 4. *Lophodermium pinastri* B

Clade 4 accommodated sequences from Australia and New Zealand from five different *Pinus* spp. The ITS analysis revealed three different haplotypes (Supplementary Table S1), two haplotypes present only in Australia and one haplotype

shared between Australia and the New Zealand sequences obtained from the direct PCR of the herbarium material (Fig. 1). This clade also included the sequence AF013224 from an ex-type isolate of *L. pinastri* (ATCC 28347) from Scotland (Minter et al., 1978) which has been used as a reference sequence for *L. pinastri* in previous studies (Prihatini et al., 2016). Additionally, this clade included other sequences of *L. pinastri* from countries such as Finland, Poland, Germany, Scotland and Australia (Fig. 1). Analyses of the *ACT* sequences revealed four unique haplotypes (Supplementary Table S1) for the isolates from Australia that grouped with sequences of *L. pinastri* from Scotland with a bootstrap support of 100% (Fig. 2). Similarly, the Australian isolates from clade 4 resulted in three haplotypes in the *TEF*1 analyses (Supplementary Table S1) that were also well supported in the tree and closely related to *L. pinastri* previously collected in Australia (Fig. 3). For the purpose of this study, this clade is identified as *L. pinastri* B.

Clade 5. *Lophodermium molitoris*

A single isolate of *Lophodermium molitoris* isolated from *P. radiata* collected from New Zealand was reconfirmed in this study and was accommodated in clade 5. For the ITS analysis, this isolate grouped with sequences of *L. molitoris* previously collected in New Zealand and USA with a support of 100% (Fig. 1). For the *ACT* and *TEF*1 analyses, the isolate from this study grouped with sequences from USA and New Zealand, respectively, with a high bootstrap support (Fig. 2,3).

3.4. Morphological observations

Several features were studied to determine whether morphological distinctions could be made between the isolates from the different clades (see descriptions below). The external appearance of the ascomata was highly variable across the different hosts analysed and even for isolates within the same clade (Table 2). However, in all cases the ascomata were mainly abaxial, positioned between two rows of stomata and had a longitudinal slit down the middle, parallel with the length of the needle. The internal features studied by a cross-section through a mature ascoma revealed that the species have either a sub-hypodermal or sub-epidermal ascomata position. The exception was those of the *L. conigenum-australe* complex which showed both ascomata positions depending on the host analysed. The number of epidermal cells displaced, and their arrangement, varied between the different *Lophodermium* spp.

studied. Asci for all the species matured sequentially and were cylindrical in shape, the apex acute to sub-papillate and the walls were thin. The shape and colour for the asci and ascospores were similar for all the clades analysed, however, variation in size was observed within and between the different species. The ascospores were filiform, with a subulate apex and rounded base, hyaline, and surrounded by a gelatinous sheath.

For all isolates, cultures were initially white and over time the culture morphology became highly variable between and within the species, especially for isolates belonging to the *L. conigenum-australe* complex and *L. indianum*. Isolates grown on MEA were either lax with fluffy, aerial mycelium or compact with lobate margins. Conidia were rarely observed on MEA. Isolates grown on FGPNA were all fluffy with aerial mycelium and conidia were abundantly produced in all species, apart from *L. indianum*. The conidia produced *in vitro* on FGPNA at 8 - 10 weeks were bacilliform to oblong with rounded ends, hyaline and had smooth walls.

Clade 1*. Lophodermium conigenum-australe complex* — Fig. 4

Ascomata were thin, elongated or elliptical, black, with a surround concolorous (Fig. 4A) to the needle surface or lighter (Fig. 4 B-D). Size of the ascomata varied depending on the host and location. Ascomata observed on the needles of *Pinus elliottii x P. caribaea*, *P. oocarpa* Schiede ex Schltdl*.* and *P. radiata* were the largest and measured 795.67 to 1497.78 × 117.03 to 405.34 µm (1135.26 µm × 263.95 µm). However, the size of the ascomata observed on the needles of *Pinus elliottii x P. caribaea* in Australia were larger for the Grafton location as compared to Queensland. The size of the ascomata observed from Queensland were similar in size to the ascomata from *Pinus elliottii x P. caribaea* observed in South Africa and measured 493.55 to 935.60 × 74.99 to 235.92 µm (720.76 µm × 136.39 µm). The smallest ascomata were observed on *P. maximinoi* Moore*,* 289.86 to 491.37 × 57.98 to 84.97 µm (366.19 µm × 74.14 µm). The ascomata perimeter line may be absent, or the lines may be solid (Fig. 4A) or broken (Fig. 4C) as observed on *P. radiata* and *P. oocarpa*, respectively. All ascomata were pointed at the ends, only raised the needle surface slightly when wet and the slit of the clypeus was either grey or absent. No zone lines were observed on the needle surface for any of the hosts examined.

A midpoint vertical section showed the sub-hypo- (Fig. 4E) or sub-epidermal (Fig. 4G) position of the ascomata, depending on the host, and in both cases the

clypeus did not extend until the basal wall, which may or may not be well-developed. Examination of the sub-hypodermal position of the ascomata in the hybrid *P. elliottii x P. caribaea* revealed hypodermal cells grouped at the base with at least 2 epidermal cells grouped above (Fig. 4F). In contrast, cross-sections through the sub-epidermal ascomata of *P. elliottii* Engelm., revealed no hypodermal cells and only two epidermal cells were scattered at the base (Fig. 4H). Two different size ranges were observed for the asci and ascospores. Asci (Fig. 4I) isolated from *P. elliottii x P. caribaea* from Grafton, Australia measured at 92.20 to 126.10 × 9.40 to 11.80 µm (109.11 µm × 10.84 µm) and the ascospores (Fig. 4J) measured at 70.60 to 85.60 × 2.00 to 3.80 µm (76.92 µm × 2.78 µm). In contrast, asci isolated from *P. elliottii x P. caribaea* from Queensland, Australia and South Africa and *P. oocarpa* from South Africa measured at 40.97 to 72.74 × 5.30 to 8.407 µm (55.35 µm × 6.86 µm) and ascospores measured at 30.38 to 43.66 × 1.00 to 1.76 µm (39.56 µm × 1.51 µm).

Culture morphology was similar across all the isolates, but colour was highly variable. On MEA, all colonies were lax and fluffy, slightly raised, circular in shape with filiform margins terminating before or at the edges of the plate. Culture colour varied between the different isolates from white with no discolouration in the agar to tan or brown with a slight brown discolouration in the agar (Fig. 4K). Conidia were rarely observed on MEA. On FGPNA, colonies remained white (Fig. 4L) and no discolouration was observed in the agar. Conidia were produced in black conidial masses (Fig. 4M) and were abundant on FGPNA (Fig. 4N). The largest conidia were observed for the isolate from New Zealand, 6.50 to 8.50 × 1.00 to 1.40 µm (7.20 µm × 1.2 µm) (Fig. 4N), whereas South Africa had the smallest conidia 3.45 to 4.58 × 0.59 to 0.87 µm (3.80 µm × 0.76 µm). Conidia observed on the cultures from Australia varied in size, 2.87 to 7.35 × 0.48 to 0.98 µm (5.27 µm × 0.71 µm), and also tended to be more filiform than bacilliform in shape. No conidia were observed in the single culture from Colombia.

Clade 2. *Lophodermium indianum* — Fig. 5

Ascomata were elliptical, black, with or without a dark grey surround. A perimeter line was rarely observed around the ascomata in *P. patula* Schiede ex Schltdl. & Cham. (Fig. 5A) and *P. tecunumanii* Eguiluz & J. P. Perry (Fig. 5B). However, a black perimeter line was observed around the ascomata on the needles of *P. maximinoi* (Fig. 5C). Sizes of the ascomata on the needle surface did not change

between the different Mesoamerican *Pinus* spp. analysed and measured 721.00 to 1499.04 × 177.55 to 448.95 µm (1092.17 µm × 303.74 µm). Ascomata were pointed at the ends, in some cases slightly raised the needle surface and the colour of the slit at the clypeus was grey (Fig. 5A) or black (Fig. 5C). No zone lines were observed on any of the needles examined.

A midpoint vertical section showed the ascomata positioned sub-hypodermal, with either hypodermal cells grouped on the basal wall (Fig. 5D-E), or epidermal cells scattered above displaced hypodermal cells (Fig. 5F-G), as observed in *P. patula* and *P. maximinoi,* respectively. The clypeus ended before reaching the basal wall and the basal wall was well defined (Fig. 5D-G). Sizes of the asci (Fig. 5H) and ascospores (Fig. 5I) did not change between the different Mesoamerican *Pinus* spp. analysed. Asci measured 37.17 to 69.43 × 5.38 to 7.76 µm (54.22 um × 6.72 um) and ascospores 29.64 to 41.17 × 1.10 to 1.96 µm (36.18 um × 1.46 um).

Culture characteristics varied in morphology and colour. On MEA, the mycelium in young colonies grew in a single direction, giving the culture a swirling appearance. After 8 - 10 weeks, colonies displayed both cultural morphologies; lax and fluffy or compact with lobate margins (Fig. 5J). Overall, colonies were circular in shape, had filiform margins, terminating before or at the edges of the plate. Colour varied from totally white to tan or brown. Some colonies revealed a tan centre with white mycelium at the margin and yellow discolouration in the agar at the circumference of the colony (Fig. 5J). Few colonies were also compact, buff in colour, with irregular margins and sepia brown discolouration was observed in the agar (Fig. 5J). Conidia were not observed on MEA. On FGPNA, colonies were white to light brown and fluffy, with filiform edges that terminated at the edges of the plate (Fig. 5K). No conidia were observed on FGPNA.

Clade 3. *Lophodermium pinastri* A — Fig. 6

Pine needles to study the features of the ascomata were only available for the samples from Chile, while all samples analysed from New Zealand were in the form of cultures. Ascomata observed on *P. radiata* were oval to elliptical, black, with a grey surround and a definite, black perimeter line. Ascomata raised the needle surface, and the ends were infrequently pointed (Fig. 6A). The size of the ascomata on *P. radiata* differed between locations in Chile. Ascomata from Biobío were slightly larger, measuring 728.39 to 825.81 × 343.10 to 441.51 µm (770.31 µm × 399.10 µm) as

compared to Los Lagos, 367.12 to 698.94 × 158.14 to 283.67 µm (510.71 µm × 225.99 µm). The colour of the slit at the clypeus was either grey or black (Fig. 6B) and abundant, thin, black zone lines were observed on the needles (Fig. 6C).

A midpoint vertical section showed the sub-epidermal position of the ascomata and the clypeus thinned as it reached the basal wall, which was not very well developed (Fig. 6D). Between 5 and 8 epidermal cells were grouped on the basal wall (Fig. 6E). The average size of the asci (6F) and ascospores (6G) did not differ between the locations. Asci measured 73.30 to 123.30 × 7.10 to 12.90 µm (97.43 µm × 10.26 µm). Ascospores measured 30.98 to 47.68 × 1.02 to 2.10 µm (38.71 µm × 1.54 µm).

Culture characteristics on MEA differed between subclade 1 and subclade 2, collected from Chile and New Zealand, respectively. Colonies from subclade 1 were compact with lobate margins, slow growing, terminating growth early, circular to irregular in form, with an umbonate surface. The colonies were white with patches of salmon to tan discolouration (Fig. 6H). Conidia were abundantly produced. Colonies from subclade 2 were compact with entire margins, terminating at the edges of the plate, circular in form, flat surface, grey-white mycelium and conidia were rarely produced (Fig. 6I). On FGPNA colonies from both subclades were lax and fluffy with white mycelium, circular to irregular in form with filiform margins (Fig. 6J, K). Colonies from subclade 1 occasionally produced black zone lines at the edges of the colony (Fig. 6J). On FGPNA, conidia were abundantly produced in black clumps with white-grey conidial masses (Fig. 6L). No differences were observed in the sizes of the conidia for the two subclades, 3.80 to 4.80 × 0.50 to 1.00 µm (3.85 µm × 0.70 µm) (Fig. 6M).

Clade 4. *Lophodermium pinastri* B — Fig. 7

The ascomata were oval to elliptical, black with a grey, black or concolorous surround and a black perimeter line (Fig. 7A-B). The ascomata raised the needle surface and the ends were rounded (Fig. 7A-B). The average size of the ascomata did not differ between the different hosts analysed, measuring 631.91 to 954.21 × 274.50 to 433.43 µm (835.79 µm × 376.75 µm). The colour of the slit at the clypeus was mostly black or grey (Fig. 7A), except for *P. patula*, in which the lips appeared red (Fig. 7B). Abundant, thin, black zone lines were observed on the needle surface for all hosts analysed (Fig. 7C).

A midpoint vertical section showed the sub-epidermal position of the ascomata and the clypeus extended until the basal wall. The epidermal cells were all grouped on the basal wall, but the number of cells displaced differed between the hosts analysed. Cross sections through the ascomata of *P. ponderosa* Douglas ex Lawson revealed 4 epidermal cells displaced (Fig. 7D-E) and 9 to 11 cells were displaced in *P. nigra* Arnold (Fig. 7F-G) and *P. patula* (not shown). Asci (Fig. 7H) and ascospore (Fig. 7I) sizes varied between the different hosts. Asci from *P. ponderosa* measured 84.70 to 113.10 × 7.00 to 10.30 µm (95.28 µm × 8.93 µm), which were smaller than the asci from *P. nigra*, 113.50 to 142.00 × 7.40 to 11.50 µm (123.01 µm × 10.13 µm). Similarly, the ascospores from *P. ponderosa* were 45.78 to 62.81 × 1.73 to 1.93 µm (54.88 µm × 1.84 µm), whereas the ascospores from *P. nigra* measured 69.60 to 85.20 × 1.2 to 2.5 µm (77.4 µm × 21.75 µm).

The culture characteristics did not differ between the isolates analysed from clade 4. On MEA, colonies were compact with entire margins, terminating before the edges of the plate, circular in form, with a flat surface and straw-white mycelium (Fig. 7J). Conidia were rarely produced on MEA. On FGPNA, colonies were lax and fluffy, slightly raised, circular in form, with white mycelium, filiform margins and conidia were frequently produced (Fig. 7K). Conidia were produced as black clumps with white-grey conidial masses (Fig. 7L) and measured 3.80 to 6.80 × 1.00 to 1.60 µm (4.95 µm × 1.30 µm) (Fig. 7M).

Clade 5*. Lophodermium molitoris* — Fig. 8

Culture characteristics for *L. molitoris* on MEA showed colonies were compact with entire margins, terminating short of the edges of the plate. Colonies were circular in form with a flat surface and were brown at the centre with tan, filiform edges (Fig. 8A). Conidia were rarely produced on MEA. On FGPNA, colonies were lax and fluffy, slightly raised, with a circular to irregular form, white mycelium and filiform margins (Fig. 8B). Conidia were frequently produced in black, conidial masses, 4.10 to 5.90 × 0.80 to 1.30 µm (5.20 µm × 1.00 µm) (Fig. 8C).

Other — *Meloderma desmazierii,* an endophyte first found on *P. strobus* L. in Canada, is distinguished from species of *Lophodermium* by the ITS, *ACT* and *TEF*1 analyses. However, it falls within the same lineage as *L. indianum*. Morphologically, this species

is distinct in having shorter ascospores than species of *Lophodermium* (Supplementary material A).

4. DISCUSSION

Multigene phylogenetic analyses based on the ITS, *ACT* and *TEF*1 regions revealed the presence of at least five well supported *Lophodermium* taxa on non-native *Pinus* spp. grown in Australia, Chile, Colombia, New Zealand and South Africa (Summarised in Fig. 9). Two of these could be identified with confidence as *L. indianum* and *L. molitoris*, from Colombia and New Zealand, respectively. The remaining three species had unclear taxonomic boundaries and were identified as residing in the *Lophodermium conigenum-australe* complex, as well as *Lophodermium pinastri* A and *Lophodermium pinastri* B in this study. Isolates belonging to the *L. conigenum-australe* complex were collected from all countries, except Chile and the two distinct lineages of *L. pinastri* included isolates from Chile, New Zealand and Australia.

Isolates representing the *L. conigenum-australe* complex were confirmed using DNA sequence data for the first time in Colombia and South Africa and on the hosts *P. maximinoi, P. oocarpa* and the hybrid *P. elliottii x P. caribaea*. This species was previously reported from Chile, most likely based on morphological descriptions, on several species of pine (Butin and Peredo, 1986). However, it was not found on any of the samples collected from *P. radiata* in Chile considered in this study. The results of this study shows that features traditionally used to distinguish between *L. conigenum* and *L. australe,* such as the shape of the ascomata and the presence or absence of a pigmented basal wall (Minter, 1981) were highly variable across the different hosts analysed. Our results were consistent with those from previous studies that suggest a single species of *Lophodermium* may be morphologically variable when isolated from different hosts (Ortiz-García et al., 2003).

Lophodermium indianum, first described from India on *P. roxburghii* Sarg.*,* was shown to occur in Colombia on *P. maximinoi, P. tecunumanii* and the hybrid *P. patula x P. maximinoi.* The isolates from Colombia were morphologically characteristic of that species in that they produced no conidia as suggested by Minter (1981). However, collections of *L. indianum* examined in this study did not produce black zone lines on the infected needles, possibly suggesting that our collections might be different to the species analysed on other hosts. Isolates from Guatemala used in this study formed

a separate lineage from the isolates from Mexico and Colombia, based on the three gene regions analyses, suggesting that the species encompasses a number of cryptic taxa.

Phylogenetic analyses in recent studies have suggested that the morphological species, *L. pinastri,* represents a species complex (Koukol et al., 2015; Salas-Lizana and Oono, 2018b). Johnston et al. (2003) identified two lineages of *L. pinastri,* the first lineage was reported from Oregon and New Zealand. Results from the present study showed that these lineages also accommodate isolates collected from Chile and were closely related to isolates from East Asia. The isolates that we treated as *L. pinastri* B collected from Australia and the historical plant material obtained from the NZFRI-M in New Zealand, fall within the second lineage that accommodates material from Canada and countries at the Baltic Rim. Recently, collections representing both these lineages have also been reported in Scotland, along with a third lineage which seems to be confined to that region (Reignoux et al., 2014). These previous findings support the notion that the collections which we have assigned to *L. pinastri* A and *L. pinastri* B, collected in the Southern Hemisphere, may have been introduced from the western United States, Canada and Europe (Johnston et al., 2003). Morphologically, the *L. pinastri* lineages collected in this study were similar in the manner in which the ascomata were embedded in the needles, but they were distinct in the number of epidermal cells displaced and their culture characteristics.

Lophodermium molitoris, a species originally described on *P. banksiana* in Canada, has been confirmed in regions of the USA and New Zealand (Minter, 1981; Johnston et al., 2003; Ortiz-García et al., 2003). After the first report of this species on *P. radiata* in Auckland, New Zealand by Johnston et al. (2003), its presence has also been confirmed on the same host in Bay of Plenty (Salas-Lizana and Oono, 2018b). Results from the current study suggest that, in the Southern Hemisphere, *L. molitoris* is confined to New Zealand, despite *P. radiata* being planted in other countries from this region.

The *Lophodermium* spp. isolated in the present study are known to be omnipresent endophytes of pine needles across Europe and North America (Ortiz-García et al., 2003; Salas-Lizana and Oono, 2018b; Bartnik et al., 2021). An important outcome from this study is that the pathogenic species, *L. seditiosum*, was not isolated from any of the countries sampled, despite the species being reported from Chile and South Africa in the past (Roux and Lundquist, 1984; Butin and Peredo, 1986). This

could suggest that previous reports of the pathogen in these countries were incorrect, especially given the morphological overlap between the pathogenic species and the common endophyte, *L. conigenum* (Minter, 1980). This supports the need for correct diagnosis by combining molecular data with the morphological data.

The distribution observed for the species in each of the countries suggests that *Lophodermium* spp. have not been extensively moved between countries in the Southern Hemisphere where *Pinus* spp. are widely planted for commercial purposes. What was interesting in this study is that the *Lophodermium* spp. collected from Australia, Colombia, New Zealand and South Africa were from a variety of different *Pinus* spp. This was previously reported for *L. pinastri* from New Zealand, and Salas-Lizana and Oono (2018b) suggested that this could be due to a single introduction of the *Lophodermium* spp. and subsequent host diversification. However, it is also likely that multiple introduction of *Lophodermium* spp. have occurred into countries from the Southern Hemisphere in the past. It is also important to note that, based on this study, the two lineages of *L. indianum* present in Colombia, Mexico and Guatemala were not collected from any of the other countries in the Southern Hemisphere. Therefore, it is vital to reduce the likelihood that these species are introduced into the Southern Hemisphere, especially as Mesoamerican *Pinus* spp. become increasingly popular in commercial forestry.

The presence of *Lophodermium* taxa from several countries in the Southern Hemisphere was confirmed using DNA sequence data for the first time and this data can be used to enrich the database for *Lophodermium* spp. Additionally, this study showed that the *TEF*1 gene region amplified relatively well for different taxa of *Lophodermium* and that it is a promising marker for phylogenetic studies. One drawback is that several of the taxa remain ambiguous, but the advances in sequencing technology may aid in clarifying the species boundaries, and the sequence data generated in this study will greatly contribute to this. Future studies need to focus on the recollection and typification of *Lophodermium* spp. to allow for robust DNA-based identification.

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7. TABLES

Table 1. Information for isolates of *Lophodermium* spp. collected from non-native *Pinus* spp. grown in five countries in the Southern Hemisphere (including Colombia).

Taxon	Country	Region	Culture collection number[a, b, c]	Source[f]	Host	Collector	Collection date
Lophodermium conigenum-australe complex	Australia	New South Wales	CMW58458[d, e]	Needles	*Pinus radiata*	A.J. Carnegie	July 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58459[d]	Needles	*P. ponderosa*	A.J. Carnegie	July 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58461[d]	Needles	*P. radiata*	A.J. Carnegie	August 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58462[d]	Needles	*P. radiata*	A.J. Carnegie	September 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58457[d]	Needles	*P. radiata*	A.J. Carnegie	November 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58460[d, e]	Needles	*P. elliottii x P. caribaea*	A.J. Carnegie	November 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58463[d]	Needles	*P. pinaster*	A.J. Carnegie	November 2020
L. conigenum-australe complex	Australia	New South Wales	CMW58464[d]	Needles	*P. radiata*	A.J. Carnegie	November 2020
L. conigenum-australe complex	Australia	Queensland	CMW58465[d, e]	Needles	*P. elliottii x P. caribaea*	M. Ramsden & H. Nahrung	December 2020
L. conigenum-australe complex	Australia	Queensland	CMW58467[d]	Needles	*P. elliottii x P. caribaea*	M. Ramsden & H. Nahrung	December 2020
L. conigenum-australe complex	Australia	Victoria	CMW58466[d]	Needles	*P. halepensis*	D. Smith	October 2020
L. conigenum-australe complex	Colombia	Arauca Department	CMW34719[d, e]	Culture	*Pinus* sp.	M.J. Wingfield	February 2009
L. conigenum-australe complex	New Zealand	Auckland	NZFS756/CMW57336[d]	Culture	*P. radiata*	L. Renney	September 2001
L. conigenum-australe complex	New Zealand	Auckland	NZFS757/CMW57393[d]	Culture	*P. radiata*	J. Campbell	September 2001
L. conigenum-australe complex	New Zealand	Bay of Plenty	NZFS3286/CMW57342[d]	Culture	*P. radiata*	R.J. Ganley	November 2008
L. conigenum-australe complex	New Zealand	Bay of Plenty	NZFS707/CMW57338[d]	Culture	*P. radiata*	Unknown	Unknown
L. conigenum-australe complex	New Zealand	Taupo	NZFS694/CMW57339[d]	Culture	*P. patula*	L. Renney	July 2001
L. conigenum-australe complex	New Zealand	Taupo	NZFS708/CMW57337[d]	Culture	*P. contorta*	L. Renney	August 2001
L. conigenum-australe complex	New Zealand	Taupo	NZFS781/CMW57334[d]	Culture	*P. radiata*	J.A. Bartram	October 2001

L. conigenum-australe complex	New Zealand	Taupo	NZFS787/CMW57332	Culture	*P. radiata*	C.W. Barr	August 2001
L. conigenum-australe complex	New Zealand	Taupo	NZFS822/CMW57326	Culture	*P. ponderosa*	L. Renney	October 2001
L. conigenum-australe complex	New Zealand	Taupo	NZFS370/CMW57340[d, e]	Culture	*P. radiata*	J. Pascoe	April 2000
L. conigenum-australe complex	South Africa	Eastern Cape	CMW58744[d, e]	Needles	*P. maximinoi*	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Eastern Cape	CMW58745[d, e]	Needles	*P. maximinoi*	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58490[d]	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58491[d]	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58488[d]	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58489	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58501	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58502	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58503	Needles	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58505[d, e]	Needles	*P. elliottii*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54460	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54461[d]	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54462	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54463[e]	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54464[d]	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54465[d]	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54466	Culture	*P. patula*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54467	Culture	*P. elliottii x P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58506[d, e]	Needles	*P. oocarpa*	C.A. Rodas, M.J. Wingfield	May 2019
L. conigenum-australe complex	South Africa	Western Cape	CMW58492[d]	Needles	*P. radiata*	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58493[d]	Needles	*P. radiata*	B. Hurley, B. Slippers & D. Herron	August 2020

L. conigenum-australe complex	South Africa	Western Cape	CMW58494[d, e]	Needles	*P. radiata*	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58495[e]	Needles	*P. radiata*	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58497[d]	Needles	*Pinus* sp.	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58498	Needles	*Pinus* sp.	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58499	Needles	*Pinus* sp.	B. Hurley, B. Slippers & D. Herron	August 2020
L. conigenum-australe complex	South Africa	Western Cape	CMW58504[e]	Needles	*P. elliottii*	M.J. Wingfield	May 2021
Lophodermium conigenum	Guatemala	Unknown	CMW39040[d]	Culture	*P. tecunumanii*	I. Barnes	June 2011
L. indianum	Colombia	Cundinamarca Department	CMW55979[d]	Culture	*P. patula* x *P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55980[d]	Culture	*P. patula* x *P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55981	Culture	*P. patula x P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55982[c]	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55983	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55984[d]	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55985	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55986[d]	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55987[d, e]	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55988[d]	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020

L. indianum	Colombia	Cundinamarca Department	CMW55989	Culture	*P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Putumayo Department	CMW56849[d]	Needles	*P. tecunumanii*	C.A. Rodas	February 2020
L. indianum	Colombia	Risaralda Department	CMW58480[e]	Needles	*P. patula*	M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW58484	Needles	*P. maximinoi*	M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW58485[d]	Needles	*P. patula x P. tecunumanii*	M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW56845[d]	Needles	*P. patula x P. tecunumanii*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56846[e]	Needles	*P. maximinoi*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56847[e]	Needles	*P. tecunumanii*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58481[e]	Needles	*P. tecunumanii*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56848	Needles	*P. maximinoi*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58482	Needles	*P. maximinoi*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58483	Needles	*P. maximinoi*	M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56852	Needles	*P. maximinoi*	M.J. Wingfield	February 2020
L. indianum	Colombia	Unknown	CMW56850[d, e]	Needles	*P. maximinoi*	M.J. Wingfield	February 2020
L. indianum	Colombia	Unknown	CMW56853	Needles	*P. maximinoi*	M.J. Wingfield	February 2020
L. indianum	Colombia	Unknown	CMW56851[e]	Needles	*P. maximinoi*	M.J. Wingfield	February 2020
L. indianum	Guatemala	San Jerónimo	CMW58486[d]	Culture[d]	*P. tecunumanii*	I. Barnes	June 2011
L. indianum	Guatemala	San Jerónimo	CMW58487[d]	Culture	*P. tecunumanii*	I. Barnes	June 2011

L. indianum	Guatemala	Unknown	CMW39036[d]	Culture	*P. maximinoi*	I. Barnes	June 2011
L. indianum	Guatemala	Unknown	CMW39037	Culture	*P. maximinoi*	I. Barnes	June 2011
L. indianum	Guatemala	Unknown	CMW39038[d]	Culture	*P. maximinoi*	I. Barnes	June 2011
L. indianum	Guatemala	Unknown	CMW39039	Culture	*P. maximinoi*	I. Barnes	June 2011
L. indianum	Guatemala	Unknown	CMW39128[d]	Culture	*P. maximinoi*	I. Barnes	June 2011
Lophodermium molitoris	New Zealand	Bay of Plenty	NZFS3288/CMW57341[d, e]	Culture	*P. radiata*	L. Bulman	October 2007
Lophodermium pinastri	Australia	New South Wales	CMW56856[d, e]	Needles	*P. patula*	A.J. Carnegie	October 2020
L. pinastri	Australia	New South Wales	CMW56857[d, e]	Needles	*P. nigra*	A.J. Carnegie	October 2020
L. pinastri	Australia	New South Wales	CMW56854[d]	Needles	*Pinus* sp.	A.J Carnegie	August 2020
L. pinastri	Australia	New South Wales	CMW56855[d, e]	Needles	*P. ponderosa*	A.J. Carnegie	August 2020
L. pinastri	Chile	Biobío	CMW58470[d, e]	Needles	*P. radiata*	R. Ahumada & R. Gómez	February 2021
L. pinastri	Chile	Biobío	CMW58471[d, e]	Needles	*P. radiata*	R. Ahumada & R. Gómez	February 2021
L. pinastri	Chile	Biobío	CMW58474[d]	Needles	*P. radiata*	R. Ahumada & R. Gómez	February 2021
L. pinastri	Chile	Los Lagos	CMW58472[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
L. pinastri	Chile	Los Lagos	CMW58475[d, e]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
L. pinastri	Chile	Los Ríos	CMW58469[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
L. pinastri	Chile	Los Ríos	CMW58473[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	February 2021
L. pinastri	Chile	Los Ríos	CMW58476[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	February 2021
L. pinastri	New Zealand	Dunedin	NZFRI-M3975	Direct PCR	*P. radiata*	M. Smith	November 1998
L. pinastri	New Zealand	Nelson	NZFRI-M4654	Direct PCR	*P. wallichiana*	R.F. Thum	March 1983
L. pinastri	New Zealand	Taranaki	NZFS801/CMW57330[d, e]	Culture	*P. densiflora*	B.J. Rogan	October 2001
L. pinastri	New Zealand	Taranaki	NZFS803/CMW57329	Culture	*P. palustris*	B.J. Rogan	October 2001
L. pinastri	New Zealand	Taranaki	NZFS804/CMW57328	Culture	*P. palustris*	B.J. Rogan	October 2001
L. pinastri	New Zealand	Wellington	NZFS776/CMW57335[d]	Culture	*P. roxburghii*	B.J. Rogan	September 2009
L. pinastri	New Zealand	Wellington	NZFS783/CMW57333	Culture	*P. muricata*	B.J. Rogan	September 2001
L. pinastri	New Zealand	Wellington	NZFS797/CMW57331	Culture	*P. sargentii*	B.J. Rogan	September 2001
L. pinastri	New Zealand	Wellington	NZFS819/CMW57327	Culture	*P. palustris*	B.J. Rogan	October 2001

Meloderma desmazierii	Chile	Aruacanía	CMW58479[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
M. desmazierii	Chile	Biobío	CMW58477[e]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
M. desmazierii	Chile	Biobío	CMW58478	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021
M. desmazierii	Chile	Los Ríos	CMW58468	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021

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

[b] NZFS, National Forest Culture Collection, Scion, New Zealand.

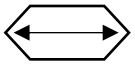
[c] NZFRI-M, National Forestry Herbarium and Xylarium, Scion, New Zealand.

[d] Isolates used in phylogenetic analyses.

[e] Samples used in morphological analyses.

[f] Isolates were obtained either directly from isolations made from the ascocarp on needles or requested as cultures from culture collections. If cultures could not be obtained, a direct PCR was done from the herbarium material in order to obtain an identification. Samples from Australia are logged in the NSW Plant Pathology and Mycology Herbarium (DAR).

Table 2. Comparative morphological features of the ascocarp and cultures for four of the phylogenetic clades presented by *Lophodermium conigenum-australe* complex, *L. indianum, L. pinastri* A and *L. pinastri* B collected in this study compared to the published descriptions for the known species by Minter (1981).

Feature	Clade 1. *L. conigenum-australe* complex	*L. conigenum* [a]	*L. australe* [a]	Clade 2. *L. indianum*	*L. indianum* [a]	Clade 3. *L. pinastri* A	Clade 4. *L. pinastri* B	*L. pinastri* [a]	*L. seditiosum* [a]
Ascomata external features									
Shape and colour	Thin, elongated, or elliptical Black	Elliptical or elongated Black	Thin, acute Black	Elliptical Black	Elliptical Black	Oval to elliptical Black	Oval to elliptical Black	Elliptical Black	Elliptical Grey to black
Ascocarp surround	Concolorous to the needle surface or lighter	Grey	Faint grey	Absent or dark grey	Grey or concolorous to the needle surface	Grey	Grey	Grey	Grey
Perimeter line	Absent, broken, or solid black lines Pointed at the ends	Black Pointed at the ends	Absent	Absent or black Pointed at the ends	Black Pointed at the ends	Black Rounded	Black Rounded	Black Rounded	Black Pointed at each end
Size (µm) 	289.86 — 491.37 (366.19) / 493.55 — 935.60 (74.14) / 795.67 — 1497.78 (1135.26)	900 — 2000	500 — 2000	721.00 — 1499.04 (1092.17)	700 — 1100	367.12 — 698.94 (510.71) / 728.39 — 825.81 (770.31)	631.91 — 954.21 (835.79)	700 — 1200	800 — 1600
Slit colour of the clypeus	Absent or grey	Grey, green, fawn or occasionally red	Grey	Grey or black	Grey	Grey or black	Grey or black, infrequently red	Grey, red, orange, yellow or rarely green	Grey, blue or green
Zone lines	Absent	Brown, infrequent	Absent	Absent	Black, infrequent	Black, frequent	Black, frequent	Black, frequent	Brown, infrequent

Ascomata internal features

Ascomata position	Sub-hypodermal or sub-epidermal	Sub-epidermal	Sub-hypodermal or Sub-epidermal	Sub-hypodermal	Sub-hypodermal	Sub-epidermal	Sub-epidermal	Subepidermal	Sub-epidermal
Hypodermal cells displaced	Yes	No	Yes	Yes	Yes	No	No	No	No
Number and arrangement of displaced epidermal cells	2, grouped or scattered	<7, scattered	<5, scattered	0-2, scattered	0-4, scattered	5-8, grouped	4 or 9-11, grouped	>5, grouped	None
Basal wall	Well developed	Poorly developed	Well developed	Well developed	Well developed	Poorly developed	Poorly developed	Poorly developed	Poorly developed
Ascus (length x width µm)	70.60 — 85.60 (76.92) x 2.00 — 3.80 (2.78) / 92.20 — 126.10 (109.11) x 9.40 — 11.80 (10.84)	160.00 —215.00 x 11.50 — 14.00	65.00 — 150.00 x 8.00 — 12.00	37.17 — 69.43 (54.22) x 5.38 — 7.76 (6.72)	80.00 — 130.00 x 9.00 -12.00	73.30 — 123.30 (97.43) x 7.10 — 12.90 (10.26)	84.70 — 113.10 (95.28) x 7.00 — 10.3 (8.93) / 113.5 — 142.00 (123.01) x 7.40 — 11.50 (10.13)	110.00 — 155.00 x 9.5.00 — 11.50	140.00 — 170.00 x 11.00 — 13.50

Ascospores (length x width µm)	40.97 — 72.74 (55.35) x 5.30 — 8.41 (6.86) / 30.38 — 43.66 (39.56) x 1.00 — 1.76 (1.51).	90.00 — 130.00 x 2.00	55.00 — 120.00 x 2.00	29.64 — 41.17 (36.18) x 1.10 — 1.96 (1.46)	70.00 — 200.00 x 2.00	30.98 — 47.68 (38.71) x 1.02 — 2.10 (1.54)	45.78 — 62.81 (54.88) x 1.73 — 1.93 (1.84) / 69.60 — 85.20 (77.42) x 1.2 — 2.5 (1.75)	70.00 — 110.00 x 2.00	90.00 — 120.00 x 2.00
Culture characteristics (MEA)									
Morphology	Fast-growing, lax, fluffy	Fast-growing, lax, fluffy	Fast-growing, lax, fluffy	Fast-growing, lax, fluffy or slow-growing, compact, lobate	NA	Fast-growing, lax, flat or slow-growing, compact	Slow growing, compact	Slow growing, compact	Fast growing, lax, fluffy
Colour	White, tan, or brown	White, tan or occasionally brown	White, tan, or brown	White, tan, both or brown only	NA	White with tan patches or straw-white	Straw-white, occasionally black zone line on circumference	White, thin, black zone line on circumference	White, tan, or brown
Pigments	Brown	Brown	No pigmentation	Yellow to dark brown	NA	No pigmentation	No pigmentation	No pigmentation	Brown

Conidia (length x width µm)	3.45 — 4.58 (3.80) x 0.59 — 0.87 (0.76) / 6.50 — 8.50 (7.20) x 1.00 — 1.40 (1.2) / 2.87 — 7.35 (5.27) x 0.48 — 0.98 (0.71)	4.50 — 9.50 x 1.00	6.25 — 9.00 x 1.00	Not observed	No conidial stage	3.80 — 4.80 (3.80) x 0.50 — 1.00 (0.70)	3.80 — 6.80 (4.95) x 1.00 — 1.60 (1.30)	4.50 — 6.25	6.00 — 8.00 x 1.00

[a] Minter (1981).

Abbreviation NA, not available.

8. FIGURES


Key:
Australia (AUS)
Chile (CHL)
Colombia (COL)
New Zealand (NZL)
South Africa (ZAF)
Clade
L. conigenum-australe complex
1
L. indianum
2
Subclade 1
Subclade 2
L. pinastri A
3
Subclade 1
Subclade 2
L. pinastri B
4
L. molitoris
5
0.04

Fig. 1. Maximum likelihood tree based on the internal transcribed spacer (ITS) region for representative *Lophodermium* species and related taxa. Isolates generated in this study from five countries located in the Southern Hemisphere (including Colombia), indicated according to key, fall within five clades of recognized *Lophodermium* species. Ex-type specimens are indicated with a “T” and additional sequences generated in this study are indicated in bold. *Colpoma quercinum* and *Tryblidiopsis pinastri* were used as the outgroup taxa. Numbers at the nodes indicate the bootstrap support (*n* = 1,000) and the posterior probabilities from Bayesian inference, respectively.

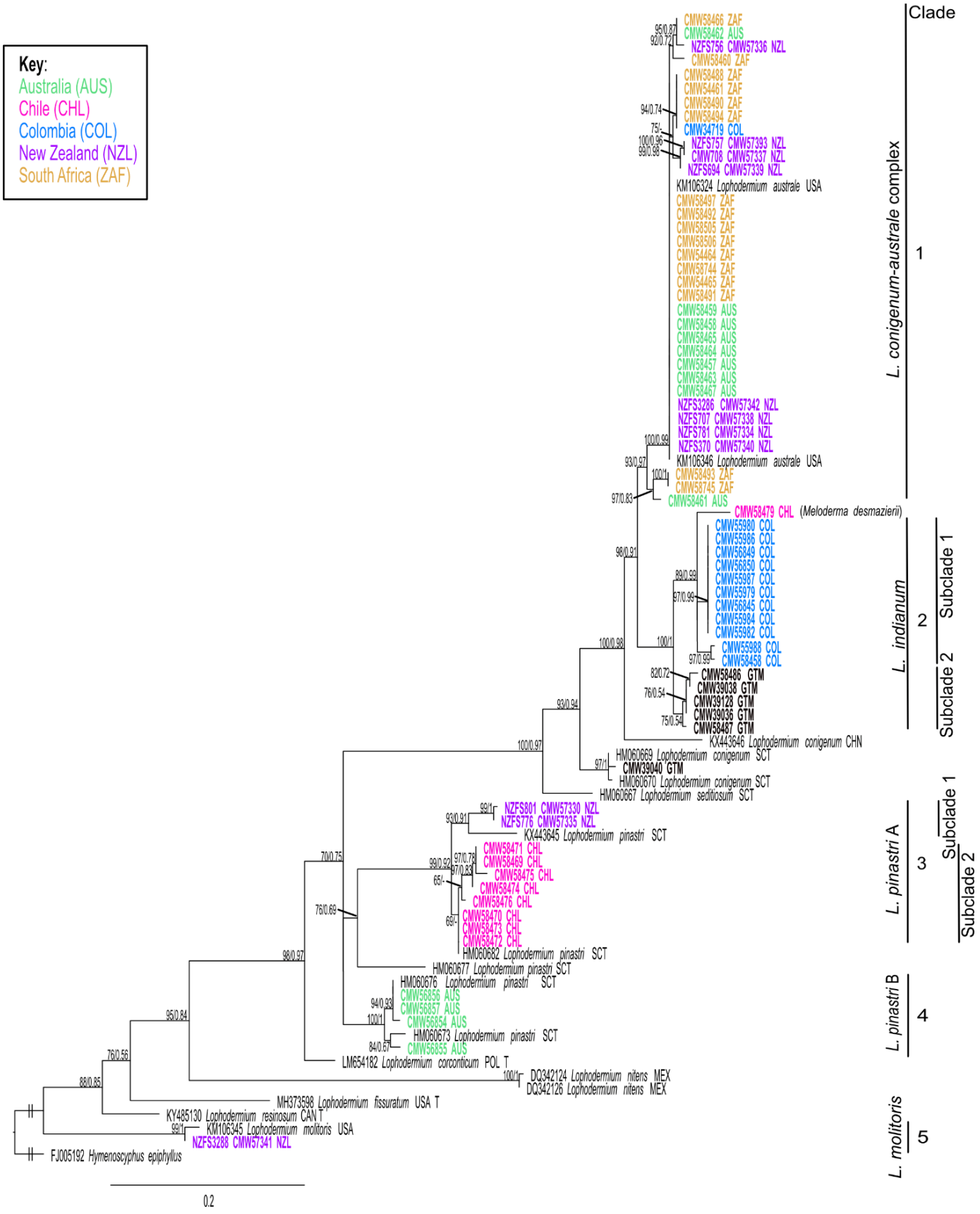

Key:
Australia (AUS)
Chile (CHL)
Colombia (COL)
New Zealand (NZL)
South Africa (ZAF)
Clade
L. conigenum-australe complex
1
L. indianum
2
Subclade 1
Subclade 2
L. pinastri A
3
Subclade 1
Subclade 2
L. pinastri B
4
L. molitoris
5
CMW58466 ZAF
CMW58462 AUS
NZFS756 CMW57336 NZL
CMW58460 ZAF
CMW58488 ZAF
CMW54461 ZAF
CMW58490 ZAF
CMW58494 ZAF
CMW34719 COL
NZFS757 CMW57393 NZL
CMW708 CMW57337 NZL
NZFS694 CMW57339 NZL
KM106324 Lophodermium australe USA
CMW58497 ZAF
CMW58492 ZAF
CMW58505 ZAF
CMW58506 ZAF
CMW54464 ZAF
CMW58744 ZAF
CMW54465 ZAF
CMW58491 ZAF
CMW58459 AUS
CMW58458 AUS
CMW58465 AUS
CMW58464 AUS
CMW58457 AUS
CMW58463 AUS
CMW58467 AUS
NZFS3286 CMW57342 NZL
NZFS707 CMW57338 NZL
NZFS781 CMW57334 NZL
NZFS370 CMW57340 NZL
KM106346 Lophodermium australe USA
CMW58493 ZAF
CMW58745 ZAF
CMW58461 AUS
CMW58479 CHL (Meloderma desmazierii)
CMW55980 COL
CMW55986 COL
CMW56849 COL
CMW56850 COL
CMW55987 COL
CMW55979 COL
CMW56845 COL
CMW55984 COL
CMW55982 COL
CMW55988 COL
CMW58458 COL
CMW58486 GTM
CMW39038 GTM
CMW39128 GTM
CMW39036 GTM
CMW58487 GTM
KX443646 Lophodermium conigenum CHN
HM060669 Lophodermium conigenum SCT
CMW39040 GTM
HM060670 Lophodermium conigenum SCT
HM060667 Lophodermium seditiosum SCT
NZFS801 CMW57330 NZL
NZFS776 CMW57335 NZL
KX443645 Lophodermium pinastri SCT
CMW58471 CHL
CMW58469 CHL
CMW58475 CHL
CMW58474 CHL
CMW58476 CHL
CMW58470 CHL
CMW58473 CHL
CMW58472 CHL
HM060682 Lophodermium pinastri SCT
HM060677 Lophodermium pinastri SCT
HM060676 Lophodermium pinastri SCT
CMW56856 AUS
CMW56857 AUS
CMW56854 AUS
HM060673 Lophodermium pinastri SCT
CMW56855 AUS
LM654182 Lophodermium conorticum POL T
DQ342124 Lophodermium nitens MEX
DQ342126 Lophodermium nitens MEX
MH373598 Lophodermium fissuratum USA T
KY485130 Lophodermium resinosum CAN T
KM106345 Lophodermium molitoris USA
NZFS3288 CMW57341 NZL
FJ005192 Hymenoscyphus epiphyllus
0.2

Fig. 2. Maximum likelihood tree based on the actin (*ACT*) region for representative *Lophodermium* species and related taxa. Isolates generated in this study from five countries located in the Southern Hemisphere, indicated according to key, fall within five clades. Ex-type specimens are indicated with a “T” and additional sequences generated in this study are indicated in bold. *Hymenoscyphus epiphyllus* was used as the outgroup taxon. Numbers at the nodes indicate the bootstrap support (*n* = 1,000) and posterior probabilities from Bayesian inference, respectively.

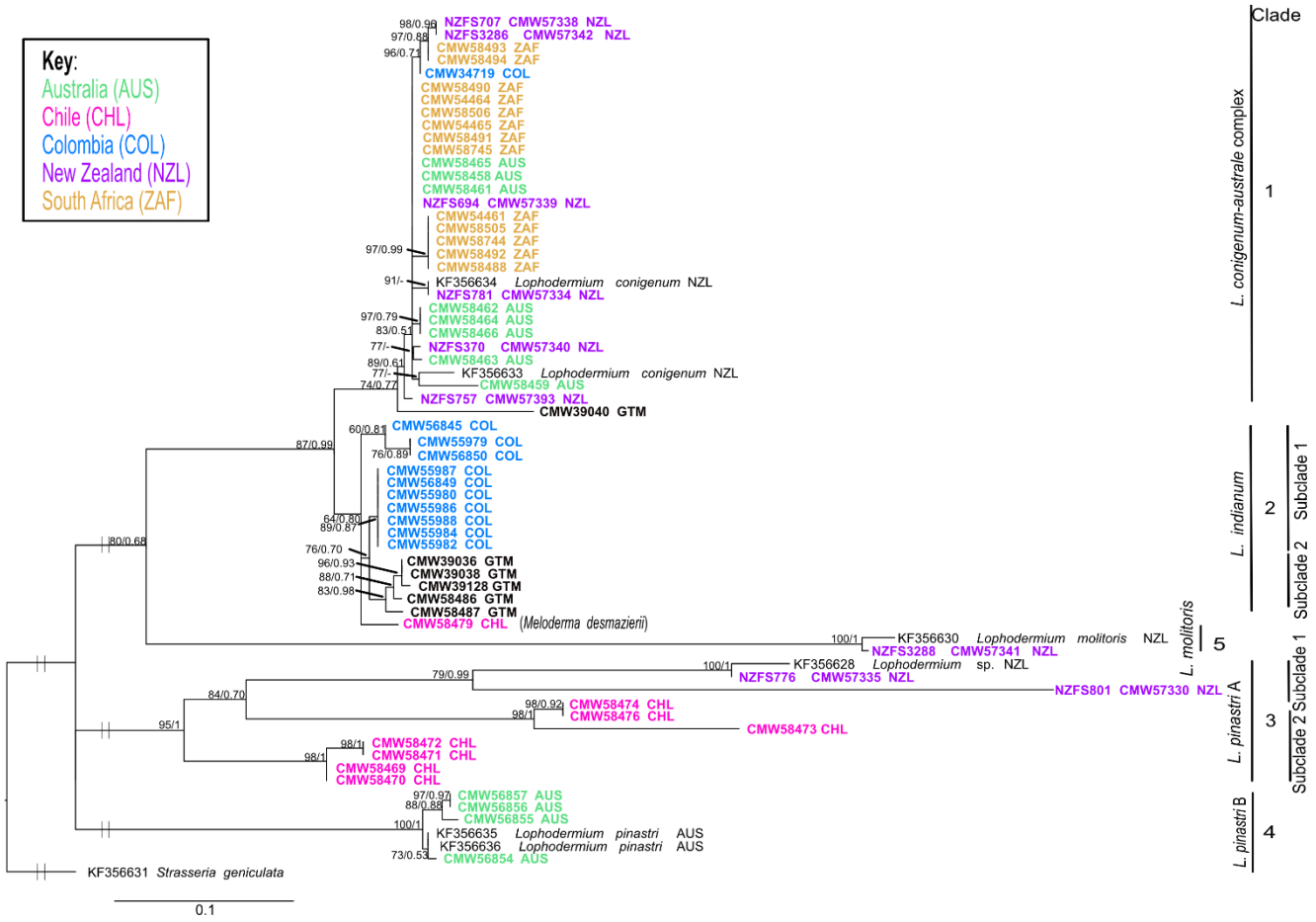


Fig. 3. Maximum likelihood tree based on the translation elongation factor 1-α (*TEF*1) region for representative *Lophodermium* species and related taxa. Isolates generated in this study from five countries located in the Southern Hemisphere, indicated according to key, fall within five clades. Additional sequences generated in this study are indicated in bold. *Strasseria geniculata* was used as the outgroup taxon. Numbers at the nodes indicate the bootstrap support (*n* = 1,000) and posterior probabilities from Bayesian inference, respectively.

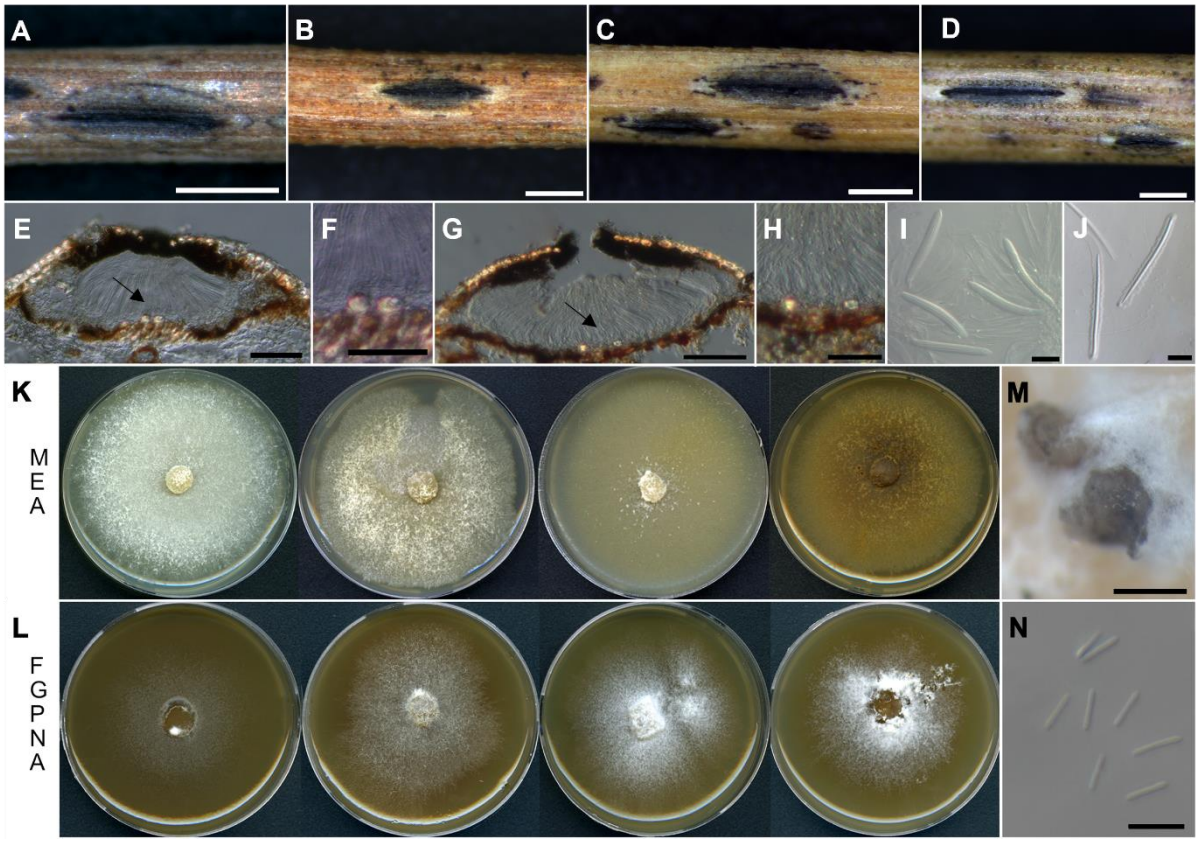


Fig. 4. Micrographs of *Lophodermium conigenum-australe* complex. Ascomata on the needle surface of **A,** *Pinus radiata* **B,** *Pinus elliottii x P. caribaea* **C,** *Pinus oocarpa* and **D,** *Pinus elliottii.* Midpoint vertical section shows the **E-F,** sub-hypodermal (*P. elliottii x caribaea*) and **G-H,** sub-epidermal position (*P. elliottii*) of mature ascoma in the needle surface and the epidermal cells displaced at the base (arrow). **I,** Asci. **J,** Ascospores. Variability observed in the culture morphology of eight-week-old colonies for isolates from South Africa (CMW54463), Australia (CMW58465), Colombia (CMW34719) and New Zealand (NZFS370/CMW57340), from left to right, grown on **K,** MEA and **L,** FGPNA. **M,** Black conidial masses produced in culture on FGPNA. **N,** Conidia. Scale bars: A-D = 500um; E-H = 50um; I = 20um; J = 10um; M = 500um; N = 10um.

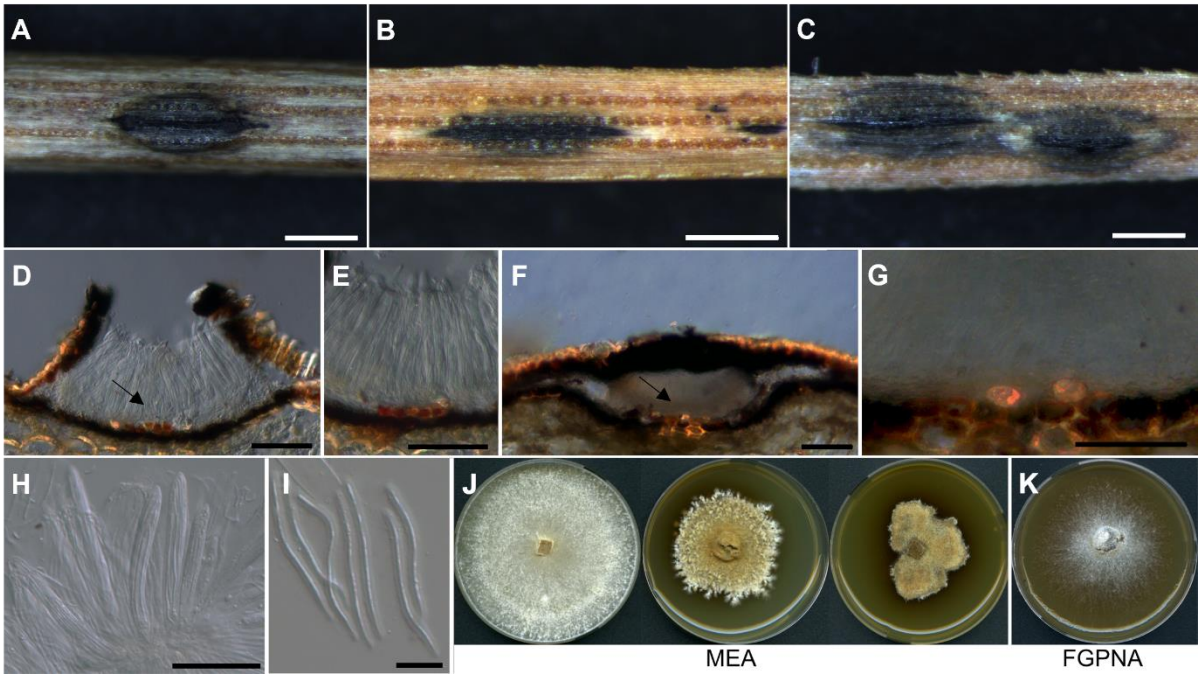


Fig. 5. Micrographs of *Lophodermium indianum* collected from Colombia. Ascomata on the needle surface of **A,** *Pinus patula* **B,** *Pinus tecunumanii* and **C,** *Pinus maximinoi.* Midpoint vertical section shows the sub-hypodermal position of the ascomata in the needle surface and the cells displaced at the base (arrow) in **D-E,** *Pinus patula* and **F-G,** *Pinus maximinoi.* **H,** Asci. **I,** Ascospores. Variability observed in the culture morphology of eight-week-old colonies for isolates (CMW55987, CMW56850 and CMW56987) grown on **J,** MEA and **K,** FGPNA. Scale bars: A-C = 500um; D-G = 50um; H = 50um; I = 10um.

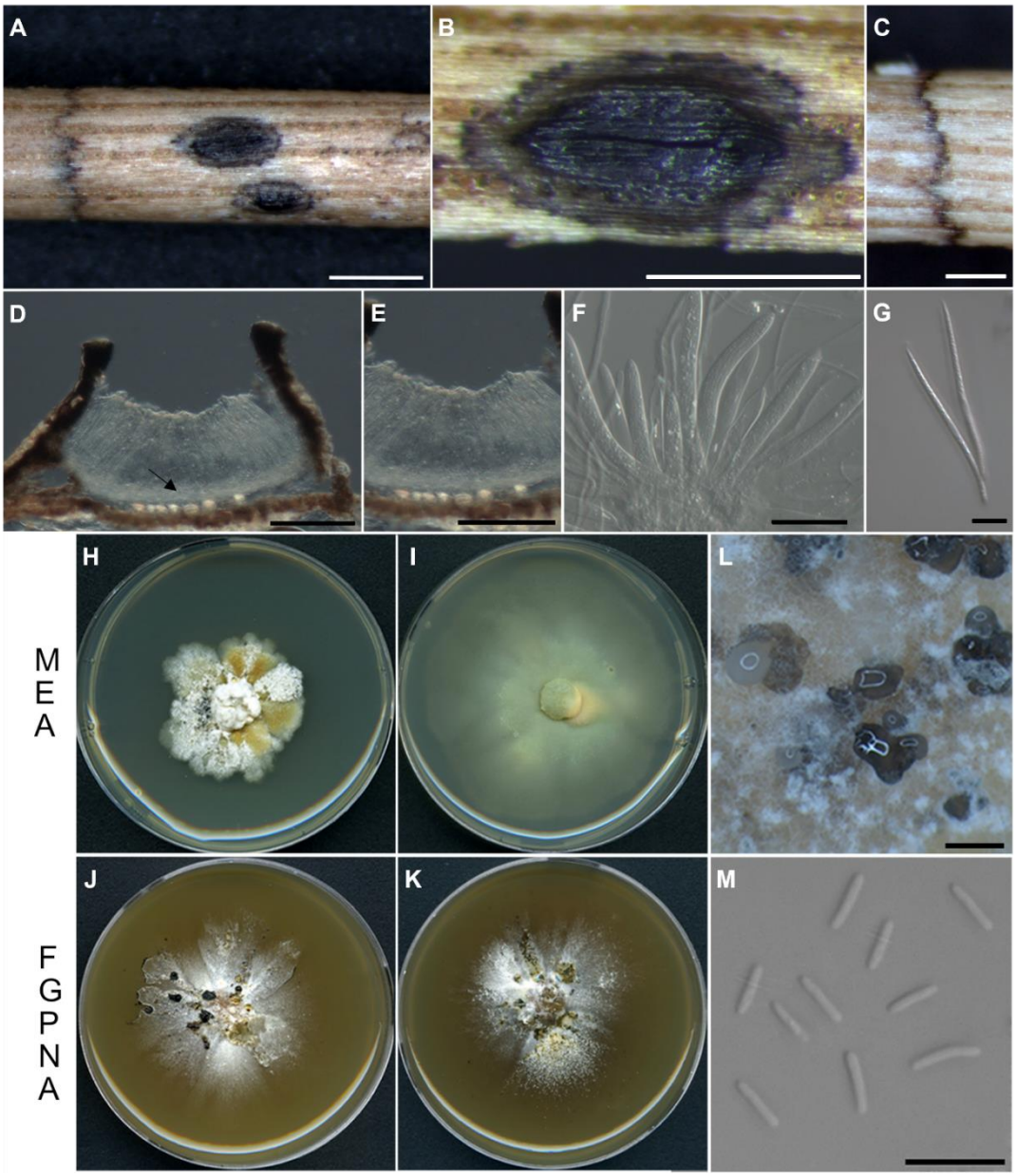


Fig. 6. Micrographs of *Lophodermium pinastri* A collected from Chile and New Zealand. **A-B,** Ascomata and **C,** black zone lines observed on the needle surface of *P. radiata*. **D-E,** Midpoint vertical section shows the sub-epidermal ascomata position in the needle surface of *P. radiata,* and the displaced epidermal cells grouped at the base (arrow). **F,** Asci. **G,** Ascospores. Variation observed in the colony morphology of eight-week-old colonies grown on **H-I,** MEA and **J-K,** FGPNA for isolates from Chile representing subclade 2 (CMW58475) and New Zealand representing subclade 1 (NZFS801/CMW57330), respectively. **L,** Conidial masses. **M,** Conidia. Scale bars: A-B = 500um; C = 200um; D = 50um; E = 20um; F = 50um ; G = 10um ; L = 500um; M = 10um.

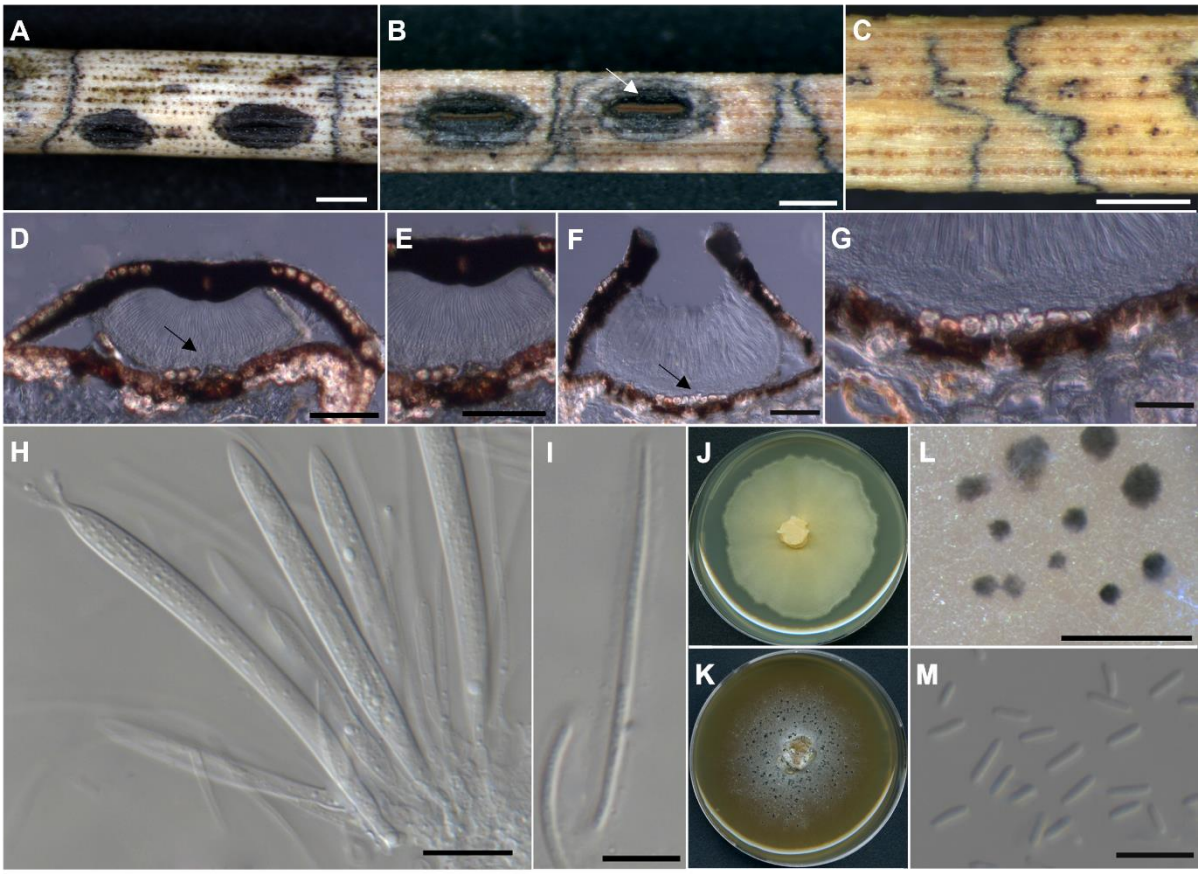


Fig. 7. Micrographs of *Lophodermium pinastri* B collected from Australia. Ascomata on the needle surface of **A,** *Pinus ponderosa* and **B,** *Pinus patula,* with black and red lips (arrow), respectively. **C,** Black zone lines observed on all samples analysed. Midpoint vertical section shows the positioning of the ascomata in the needle surface and the epidermal cells displaced at the base (arrow) in **D-E,** *Pinus ponderosa* and **F-G,** *Pinus nigra*. **H,** Asci. **I,** Ascospores. Eight-week-old colony grown on **J,** MEA and **K,** FGPNA for an isolate from Australia (CMW56855). **L,** Black conidial masses observed on the surface of FGPNA. **M,** Conidia. Scale bars: A-C= 500um; D-G = 50um; H = 20um ; I = 10um ; L = 500um; M = 10um.

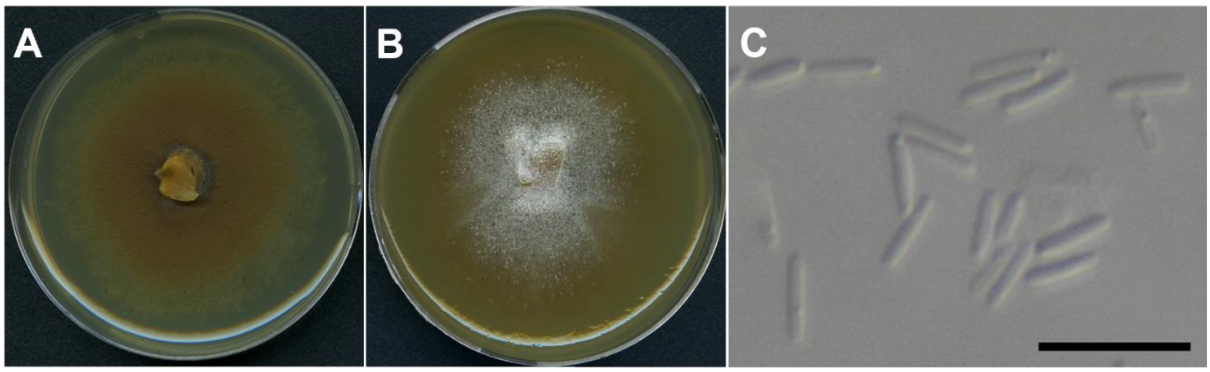


Fig. 8. Micrographs of *Lophodermium molitoris* collected in New Zealand. **A,** Eight-week-old colony grown on MEA and **B,** FGPNA, respectively (NZFS3288/CMW57341). **C,** Conidia. Scale bar: B= 10um.

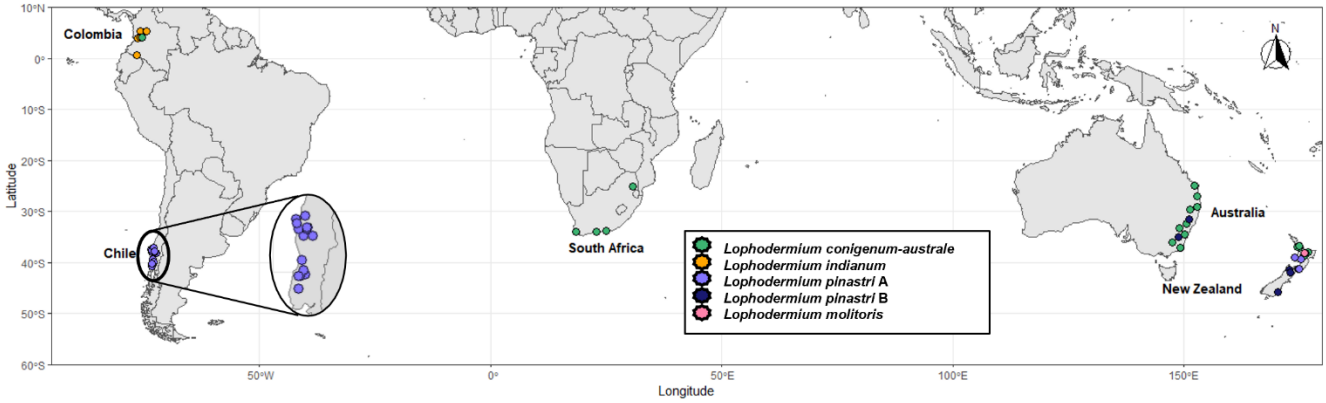


Fig. 9. Distribution of *Lophodermium* species on non-native *Pinus* spp. from Colombia, Chile, South Africa, New Zealand and Australia.

9. SUPPLEMENTARY TABLES

Supplementary Table S1. Collection information, GenBank accession numbers and haplotype information for the six taxa collected in this study.

Country	Sample ID[a, b]	CMW collection number[c]	Host	GenBank Accession Number (haplotype)		
				ITS	*ACT*	*TEF*1
Clade 1. *Lophodermium conigenum-australe* complex						
Australia[d]	AC38984	CMW58458	*Pinus radiata*	OP751779 (1)	XXX(18)	XXX(11)
Australia[d]	AC38990	CMW58459	*Pinus ponderosa*	OP751778 (1)	XXX(6)	XXX(11)
Australia[d]	AC42221	CMW58461	*Pinus radiata*	OP751776 (13)	XXX(9)	XXX(11)
Australia[d]	AC42233	CMW58462	*Pinus radiata*	OP751774 (19)	XXX(14)	XXX(7)
Australia[d]	AC2111	CMW58457	*Pinus radiata*	OP751785 (13)	XXX(19)	—
Australia[d]	AC40518	CMW58460	*Pinus caribaea x elliottii*	OP751777 (10)	XXX(4)	—
Australia[d]	AC47092	CMW58463	*Pinus pinaster*	OP751771 (1)	XXX(13	XXX(5)
Australia[d]	AC47093	CMW58464	*Pinus radiata*	OP751770 (16)	XXX(18)	XXX(7)
Australia[d]	HQP1001	CMW58465	*Pinus caribaea x elliottii*	OP751768 (9)	XXX(18)	XXX(11)
Australia[d]	HQP1002	CMW58467	*Pinus caribaea x elliottii*	OP751767 (15)	XXX(7)	—
Australia[d]	AgVic1001	CMW58466	*Pinus halepensis*	OP751769 (12)	XXX(14)	XXX(7)
Colombia[d]	LM1	CMW34719	*Pinus* sp.	*KF636500* (14)	XXX(17	XXX(2)
New Zealand[d]	NZFS756	CMW57336	*Pinus radiata*	OP751720 (4)	XXX(11)	—
New Zealand[d]	NZFS757	CMW57393	*Pinus radiata*	OP751719 (1)	XXX(12)	XXX(4)
New Zealand[d]	NZFS3286	CMW57342	*Pinus radiata*	*KY576873* (8)	XXX(8)	XXX(8)
New Zealand[d]	NZFS707	CMW57338	*Pinus radiata*	OP751722 (11)	XXX(3)	XXX(8)
New Zealand[d]	NZFS694	CMW57339	*Pinus patula*	*MG877438* (4)	XXX(12)	XXX(11)
New Zealand[d]	NZFS708	CMW57337	*Pinus contorta*	OP751721 (18)	XXX(12)	—

New Zealand[d]	NZFS781	CMW57334	*Pinus radiata*	OP751718 (5)	XXX(7)	XXX(3)
New Zealand	NZFS787	CMW57332	*Pinus radiata*	OP751717 (4)	XXX(2)	—
New Zealand	NZFS822	CMW57326	*Pinus ponderosa*	OP751716 (11)	XXX(8)	—
New Zealand[d]	NZFS370	CMW57340	*Pinus radiata*	OP751723 (4)	XXX(6)	XXX(6)
South Africa	Lop1b	CMW58489	*Pinus caribaea x elliottii*	OP751707 (17)	XXX(16)	—
South Africa[d]	SA4N2	CMW58744	*Pinus maximinoi*	OP751698 (7)	XXX(18)	XXX(10)
South Africa[d]	SA4N3	CMW58745	*Pinus maximinoi*	OP751697 (6)	XXX(19)	XXX(11)
South Africa[d]	Lop2a	CMW58490	*Pinus caribaea x elliottii*	OP751705 (2)	XXX(5)	XXX(11)
South Africa[d]	Lop2b	CMW58491	*Pinus caribaea x elliottii*	OP751704 (17)	XXX(10)	XXX(11)
South Africa[d]	Lop1a	CMW58488	*Pinus caribaea x elliottii*	OP751708 (17)	XXX(15)	XXX(10)
South Africa	SAB2	CMW58501	*Pinus caribaea x elliottii*	OP751693 (7)	XXX(7)	—
South Africa	SAB3	CMW58502	*Pinus caribaea x elliottii*	OP751692 (7)	XXX(7)	—
South Africa	SAB4	CMW58503	*Pinus caribaea x elliottii*	OP751691 (17)	XXX(7)	—
South Africa[d]	SAB7	CMW58505	*Pinus elliottii*	OP751690 (18)	XXX(7)	XXX(10)
South Africa	a-1	CMW54460	*Pinus caribaea x elliottii*	OP751715 (17)	XXX(7)	—
South Africa[d]	a-2	CMW54461	*Pinus caribaea x elliottii*	OP751714 (7)	XXX(16	XXX(10)
South Africa	a-3	CMW54462	*Pinus caribaea x elliottii*	OP751713 (7)	XXX(19)	—
South Africa	a-4	CMW54463	*Pinus caribaea x elliottii*	OP751712 (7)	XXX(7)	—
South Africa[d]	a-5	CMW54464	*Pinus caribaea x elliottii*	OP751711 (17)	XXX(8)	XXX(11)
South Africa[d]	a-6	CMW54465	*Pinus caribaea x elliottii*	OP751710 (7)	XXX(1)	XXX(11)
South Africa	Lop2	CMW54466	*Pinus patula*	OP751706 (17)	XXX(7)	—
South Africa	a-7	CMW54467	*Pinus caribaea x elliottii*	OP751709 (17)	XXX(7)	—
South Africa[d]	LRBR27C	CMW58506	*Pinus oocarpa*	OP751703 (3)	XXX(7)	XXX(11)
South Africa[d]	SA1N2	CMW58492	*Pinus radiata*	OP751701 (1)	XXX(7)	XXX(10)
South Africa[d]	SA1N1	CMW58493	*Pinus radiata*	OP751702 (1)	XXX(20)	XXX(9)
South Africa[d]	SA2N1	CMW58494	*Pinus radiata*	OP751700 (17)	XXX(16)	XXX(9)
South Africa	SA2N2	CMW58495	*Pinus radiata*	OP751699 (1)	XXX(16)	—
South Africa[d]	SA5N1	CMW58497	*Pinus* sp.	OP751696 (1)	XXX(5)	—

South Africa	SA5N2S1	CMW58498	*Pinus* sp.	OP751695 (7)	XXX(20)	—
South Africa	SA5N2S2	CMW58499	*Pinus* sp.	OP781694 (7)	XXX(15)	—
South Africa	SAL5	CMW58504	*Pinus elliottii*	OP751689 (17)	XXX(20)	—
4		49	10	19	20	11
Clade 2. *Lophodermium indianum*						
Colombia[d]	Lop1	CMW55979	*Pinus patula* x *P. tecunumanii*	OP751739 (23)	XXX(22)	XXX(18)
Colombia[d]	Lop2	CMW55980	*Pinus patula* x *P. tecunumanii*	OP751738 (25)	XXX(22)	XXX(12)
Colombia	Lop3	CMW55981	*Pinus patula x P. tecunumanii*	OP751737 (25)	XXX(22)	—
Colombia[d]	Lop4	CMW55982	*Pinus tecunumanii*	OP751736 (25)	XXX(27)	XXX(12)
Colombia	Lop5	CMW55983	*Pinus tecunumanii*	OP751735 (23)	XXX(22)	—
Colombia[d]	Lop6	CMW55984	*Pinus tecunumanii*	OP751734 (25)	XXX(21)	XXX(13)
Colombia	Lop7	CMW55985	*Pinus tecunumanii*	OP751733 (25)	XXX(24)	—
Colombia[d]	Lop8	CMW55986	*Pinus tecunumanii*	OP751732 (21)	XXX(22)	XXX(12)
Colombia[d]	Lop9	CMW55987	*Pinus tecunumanii*	OP751731 (21)	XXX(23)	XXX(12)
Colombia[d]	Lop10	CMW55988	*Pinus tecunumanii*	OP751730 (26)	XXX(26)	XXX(12)
Colombia	Lop11	CMW55989	*Pinus tecunumanii*	OP751729 (25)	XXX(27)	—
Colombia[d]	Col275VerN2	CMW56849	*Pinus tecunumanii*	OP751740 (22)	XXX(24)	XXX(12)
Colombia	Col11YorkN1	CMW58480	*Pinus patula*	OP751751 (30)	XXX(22)	—
Colombia	Col14ChnN1S1	CMW58484	*Pinus maximinoi*	OP751746 (22)	XXX(23)	—
Colombia	Col15YorkN1	CMW58485	*Pinus patula x P. tecunumanii*	OP751745 (22)	XXX(26)	—
Colombia[d]	Col15YorkN3	CMW56845	*Pinus patula x P. tecunumanii*	OP751744 (24)	XXX(22)	XXX(16)
Colombia	Col9BarN2	CMW56846	*Pinus maximinoi*	OP751752 (22)	XXX(22)	—
Colombia	Col12RocN1	CMW56847	*Pinus tecunumanii*	OP751754 (22)	XXX(22)	—
Colombia	Col12RocN4	CMW56848	*Pinus maximinoi*	OP751749 (22)	XXX(24)	—
Colombia	Col13BarN2S1	CMW58481	*Pinus tecunumanii*	OP751750 (22)	XXX(22)	—
Colombia	Col13BarN2S2	CMW58482	*Pinus maximinoi*	OP751748 (24)	XXX(22)	—
Colombia	Col13BarN4	CMW58483	*Pinus maximinoi*	OP751747 (22)	XXX(23)	—
Colombia	Col1BarN1	CMW56852	*Pinus maximinoi*	OP751753 (22)	XXX(24)	—

Colombia[d]	Col18BN3	CMW56850	*Pinus maximinoi*	OP751743 (29)	XXX(24)	XXX(18)
Colombia	Col19BN1	CMW56853	*Pinus maximinoi*	OP751742 (22)	XXX(22)	—
Colombia	Col20BN4	CMW56851	*Pinus maximinoi*	OP751741 (24)	XXX(24)	—
Guatemala[d]	7B	CMW39036	*Pinus maximinoi*	*KF636505* (26)	XXX(28)	XXX(15)
Guatemala	7S	CMW39037	*Pinus maximinoi*	*KF636503* (26)	XXX(28)	—
Guatemala[d]	MG4A	CMW39038	*Pinus maximinoi*	*KF636507* (26)	XXX(30)	XXX(15)
Guatemala	7Lb	CMW39039	*Pinus maximinoi*	*KF636506* (26)	XXX(31)	—
Guatemala[d]	7L	CMW39128	*Pinus maximinoi*	*KF535509* (26)	XXX(31)	XXX(15)
Guatemala[d]	SJIE	CMW58486	*Pinus tecunumanii*	OP751727 (27)	XXX(25)	XXX(17)
Guatemala[d]	SJBI	CMW58487	*Pinus tecunumanii*	OP751728 (28)	XXX(29)	XXX(19)
2		33	4	10	11	8
Clade 3. *Lophodermium pinastri A*						
Chile[d]	CL10.1	CMW58470	*Pinus radiata*	OP751760 (37)	XXX(34)	XXX(26)
Chile[d]	CL10.2	CMW58471	*Pinus radiata*	OP751759 (33)	XXX(40)	XXX(24)
Chile[d]	CL16.1	CMW58474	*Pinus radiata*	OP751757 (35)	XXX(39)	XXX(27)
Chile[d]	CL14	CMW58472	*Pinus radiata*	OP751758 (37)	XXX(36)	XXX(25)
Chile[d]	CL7	CMW58475	*Pinus radiata*	OP751761 (32)	XXX(38)	—
Chile[d]	CL17	CMW58469	*Pinus radiata*	OP751756 (35)	XXX(40)	XXX(26)
Chile[d]	CL1.2	CMW58473	*Pinus radiata*	OP751764 (36)	XXX(35)	XXX(21)
Chile[d]	CL1.1	CMW58476	*Pinus radiata*	OP751765 (34)	XXX(37)	XXX(27)
New Zealand[d]	NZFS801	CMW57330	*Pinus densiflora*	*MG877444* (38)	XXX(42)	XXX(22)
New Zealand	NZFS803	CMW57329	*Pinus palustris*	OP751781 (38)	XXX(42)	—
New Zealand	NZFS804	CMW57328	*Pinus palustris*	*KY576876* (38)	XXX(42)	—
New Zealand[d]	NZFS776	CMW57335	*Pinus roxburghii*	*MG877440* (38)	XXX(41)	XXX(23)
New Zealand	NZFS783	CMW57333	*Pinus muricata*	OP751782 (38)	XXX(42)	—
New Zealand	NZFS797	CMW57331	*Pinus sargentii*	OP751783 (38)	XXX(42)	—
New Zealand	NZFS819	CMW57327	*Pinus palustris*	OP751784 (38)	XXX(42)	—
2		15	6	7	9	7

Clade 4. *Lophodermium pinastri B*						
Australia[d]	AC32232	CMW56854	*Pinus* sp.	OP751780 (41)	XXX(44)	XXX(29)
Australia[d]	AC42229	CMW56855	*Pinus ponderosa*	OP751775 (40)	XXX(43)	XXX(28)
Australia[d]	AC43040	CMW56856	*Pinus patula*	OP751773 (39)	XXX(46)	XXX(30)
Australia[d]	AC43042	CMW56857	*Pinus nigra*	OP 751772 (41)	XXX(45)	XXX(30)
New Zealand[d]	NZFRI-M3975	—	*Pinus radiata*	OP751724 (40)	—	—
New Zealand	NZFRI-M4654	—	*Pinus griffithii*	OP751725 (40)	—	—
2		6	5	3	4	3
Clade 5. *Lophodermium molitoris*						
New Zealand[d]	NZFS3288	CMW57341	*Pinus radiata*	*KY576875* (42)	XXX(47)	XXX(30)
1		1	1	1	1	1
Meloderma desmazierii						
Chile[d]	CL6.2	CMW58479	*Pinus radiata*	OP751762 (20)	XXX(33)	XXX(31)
Chile	CL3.1	CMW58477	*Pinus radiata*	OP751763 (20)	XXX(33)	—
Chile	CL3.2	CMW58478	*Pinus radiata*	OP751766 (20)	XXX(33)	—
Chile	CL21	CMW58468	*Pinus radiata*	OP751755 (20)	XXX(33)	—
1		4	1	1	1	1

[a] NZFS, National Forest Culture Collection, Scion, New Zealand.

[b] NZFRI-M, National Forestry Herbarium and Xylarium, Scion, New Zealand.

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

[d] Isolates used in phylogenetic analyses.

[e] GenBank accession numbers for isolates generated in other studies are in italics.

Supplementary Table S2. Information and GenBank accession numbers for isolates included in phylogenetic analyses.

Species	Sample ID[a]	Country	Host	GenBank accession numbers		
				ITS	*ACT*	*TEF*1
Lophodermium australe	lopaus Oaxaca	Mexico	*Pinus pseudostrobus*	AY100647	—	—
Lophodermium australe	N/A	USA	*Pinus palustris*	U92308	—	—
Lophodermium australe	BPPrig1.16.1	USA	*Pinus rigida*	MW466485	—	—
Lophodermium australe	2V2	USA	*Pinus virginiana*	—	KM106324	—
Lophodermium australe	CBS 138816	USA	*Pinus taeda*	—	—	—
Lophodermium australe	DP SL 1C	USA	*Pinus taeda*	—	KM106346	—
Lophodermium baculiferum	mon2zem Nuevo Leon	Mexico	*Pinus montezumae*	AY100656	—	—
Lophodermium baculiferum	MEXU:FU29402	Mexico	*Pinus devoniana*	MG877417	—	—
Lophodermium baculiferum	NA	USA	*Pinus contorta*	AY100658	—	—
Lophodermium conigenum	NY241	Australia	*Pinus radiata*	KJ406948	—	—
Lophodermium conigenum	R114	China	Unknown	FJ861974	—	—
Lophodermium conigenum	HOU1227D	China	*Pinus taiwanensis*	—	KX443646	—
Lophodermium conigenum	NZFS3258	New Zealand	*Pinus radiata*	MG877429	—	—
Lophodermium conigenum	29-EF1	New Zealand	*Pinus radiata*	—	—	KF356633
Lophodermium conigenum	13-EF1	New Zealand	*Pinus radiata*	—	—	KF356634
Lophodermium conigenum	Lopr882	New Zealand	*Pinus radiata*	AY100645	—	—
Lophodermium conigenum	NZFS742	New Zealand	*Pinus contorta*	KY576874	—	—
Lophodermium conigenum	CMW39040	Guatemala	*Pinus tecunumanii*	KF636501	—	—
Lophodermium conigenum	NA	Korea	*Pinus* sp.	MK883697	—	—
Lophodermium conigenum	NA	Korea	*Pinus* sp.	MK883661	—	—

Lophodermium conigenum	Ab1	Scotland	*Pinus sylvestris*	HM060648	—	—
Lophodermium conigenum	Ab1	Scotland	*Pinus sylvestris*	—	HM060669	—
Lophodermium conigenum	N/A	Scotland	*Pinus sylvestris*	—	HM060670	—
Lophodermium conigenum	CL172	Australia	*Pinus radaita*	KJ407029	—	—
Lophodermium corconticum [T]	CCF 4868	Poland	*Pinus mugo*	LM654179	LM654182	—
Lophodermium fissuratum [T]	MEXU:FU29401	USA	*Pinus monticola*	MF581496	MH373598	—
Lophodermium indianum	MEXU:FU29403	Mexico	*Pinus patula*	MG877504	—	—
Lophodermium indianum	None	Mexico	*Pinus greggii*	AY100642	—	—
Lophodermium macci	S-171	Canada	*Pinus strobus*	AF540560	—	—
Lophodermium macci	LPoEW1_3_1	USA	*Pinus strobus*	MW466472	—	—
Lophodermium molitoris	25-EF1	New Zealand	*Pinus radiata*	—	—	KF356630
Lophodermium molitoris	NZFS789	New Zealand	*Pinus radiata*	AY247752	—	—
Lophodermium molitoris	CV1_3a	USA	*Pinus virginiana*	KM106803	KM106345	—
Lophodermium molitoris	CBS 597.84	USA	*Pinus taeda*	AY100659	—	—
Lophodermium molitoris	LPiVA3_17_2	USA	*Pinus virginiana*	MW466477	—	—
Lophodermium nitens	NB-283-2D	Canada	*Pinus resinosa*	KY485136	—	—
Lophodermium nitens	EF208	Korea	*Pinus* sp.	MK842120	—	—
Lophodermium nitens	41a3	Mexico	*Pinus strobiformis*	—	DQ342124	—
Lophodermium nitens	102C27_CP	Mexico	*Pinus strobiformis*	—	DQ342126	—
Lophodermium nitens	AK420	Mexico	*Pinus strobiformis*	MG877432	—	—
Lophodermium nitens	BPEW1_11_1	USA	*Pinus strobus*	MW466487	—	—
Lophodermium pinastri	43-EF1	Australia	Unknown	—	—	KF356635
Lophodermium pinastri	NY061	Australia	*Pinus radiata*	KJ406957	—	—

Lophodermium pinastri	NO276	Australia	*Pinus radiata*	KJ406952	—	—
Lophodermium pinastri	CL10	Australia	*Pinus radiata*	KJ407045	—	—
Lophodermium pinastri	44-EF1	Australia	Unknown	—	—	KF356636
Lophodermium pinastri	HOU1227C	China	*Pinus taiwanensis*	KX443658	KX443645	—
Lophodermium pinastri	L26pinRa	Finland	*Pinus sylvestris*	AY775701	—	—
Lophodermium pinastri	L230	Germany	*Pinus sylvestris*	AY100650	—	—
Lophodermium pinastri	DHL4	Japan	Unknown	AB511819	—	—
Lophodermium pinastri	EF116	Korea	*Pinus* sp.	MK842056	—	—
Lophodermium pinastri	NZFS755	New Zealand	*Pinus pinaster*	MG877439	—	—
Lophodermium pinastri	NZFS785	New Zealand	*Pinus pinaster*	AY247753	—	—
Lophodermium pinastri	Lp5W-19Pu	Poland	*Pinus mugo* subsp. *uncinata*	MN595073	—	—
Lophodermium pinastri [T]	IMI156241/ATCC28347	Scotland	*Pinus* sp.	AF013224	—	—
Lophodermium pinastri	Aa1	Scotland	*Pinus sylvestris*	HM060657	HM060673	—
Lophodermium pinastri	Aa8	Scotland	*Pinus sylvestris*	HM060655	HM060676	—
Lophodermium pinastri	Aa2	Scotland	*Pinus sylvestris*	HM060665	HM060682	—
Lophodermium pinastri	Aa13	Scotland	*Pinus sylvestris*	HM060659	HM060677	—
Lophodermium pinastri	loppon	USA	*Pinus ponderosa*	AY100649	—	—
Lophodermium pini-excelsae	R 108	China	*Pinus wallichiana*	FJ861988	—	—
Lophodermium resinosum [T]	DAOMC 251482	Canada	*Pinus resinosa*	KY485127	KY485130	KY702582
Lophodermium resinosum	LPiEW1_13_1	USA	*Pinus strobus*	MW466488	—	—
Lophodermium seditiosum	L32sedHy	Finland	*Pinus sylvestris*	AY775705	—	—
Lophodermium seditiosum	L225	Germany	*Pinus sylvestris*	AF203468	—	—

Lophodermium seditiosum [T]	ATCC 28345	Scotland	*Pinus sylvestris*	AF462435	—	—
Lophodermium seditiosum	Ac1	Scotland	*Pinus sylvestris*	—	HM060667	—
Lophodermium sp.	27-EF1	New Zealand	Unknown	—	—	KF356628
Meloderma desmazierii	DAOM 89807	Canada	*Pinus strobus*	KY485137	—	—
Outgroups						
Colpoma quercinum	N/A	Germany	*Quercus robur*	U92306	—	—
Hymenoscyphus epiphyllus	HB7054	Germany	Unknown	—	FJ005192	—
Strasseria geniculata	26-EF1	New Zealand	Unknown	—	—	KF356631
Tryblidiopsis pinastri	HOU 198	China	*Picea* sp.	KC312678	—	—

[T] Sequences from type material.

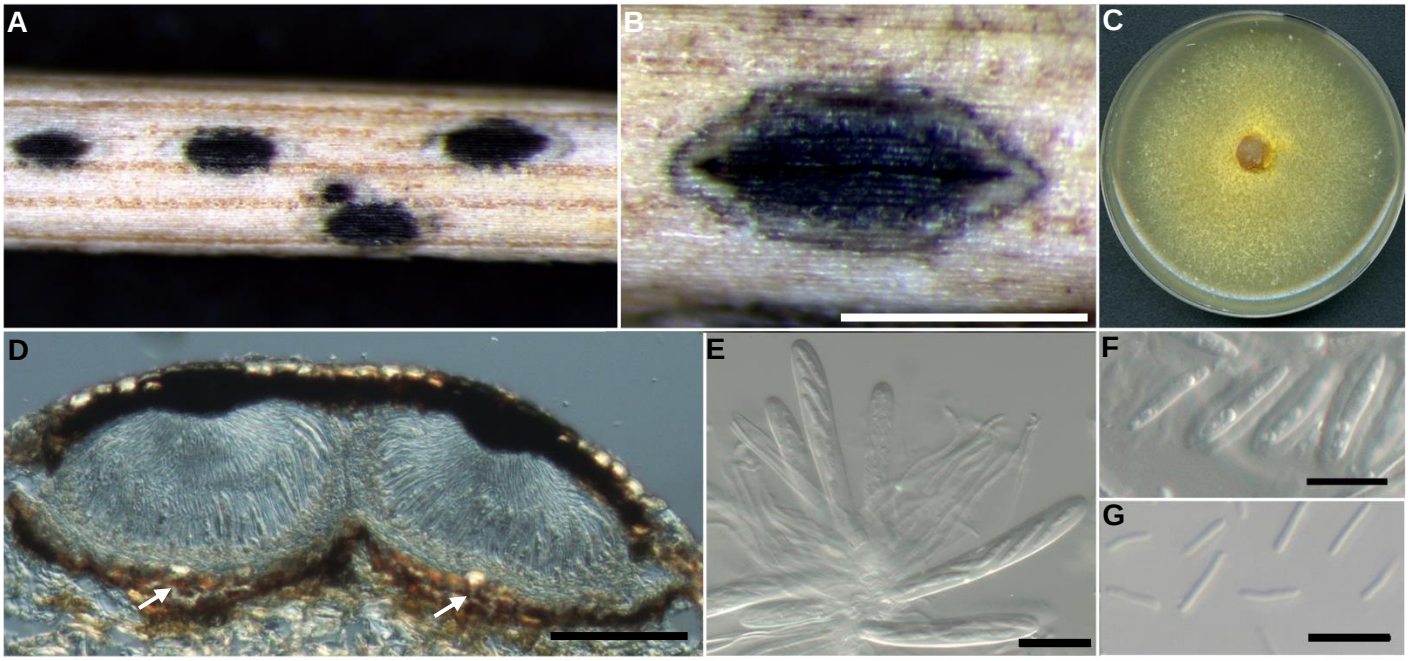
[a] Culture designations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MEXU, National Herbarium of Mexico, Universidad Nacional Autónoma de México, Distrito Federal, Ciudad Universitaria, Ciudad de México; NZFS, National Forest Culture Collection, Scion, New Zealand; CMW*,* Culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), the University of Pretoria, Pretoria, South Africa; IMI, International Mycological Institute; DAOM, Canadian National Mycological Herbarium, Ottawa Research and Development Centre, Canada; ATCC, American Type Culture Collection, Virginia, U.S.A; DAOMC, Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada.

10. SUPPLEMENTARY MATERIAL A

Meloderma desmazierii (Duby) Darker — Supplementary Fig. 1

Ascocarps were elliptical, black and lie between two rows of stomata (Supplementary Fig. 1A). The surround is concolorous to grey and there is a black perimeter line with pointed edges. No lips were present at the slit of the ascocarp. The ascocarps measured 422.10 to 772.08 × 189.50 to 255.20 µm (616.11 µm × 223.14 µm) (Supplementary Fig. 1B). No zone lines were observed on the needle surface. A midpoint vertical section through the ascocarp shows the ascocarp positioned sub-epidermal, with 4 to 6 epidermal cells grouped on the basal wall (Supplementary Fig. 1D). Asci were cylindrical, with a rounded apex, thin, smooth walls and measured 65.90 to 163.10 × 13.80 to 19.40 µm (121.30 µm × 16.50 µm) (Supplementary Fig. 1E). The ascospores were fusiform, hyaline, with a subulate apex and rounded base (Supplementary Fig. 1F). A gelatinous sheath was observed around the ascospores. The ascospores measured, 23.00 to 55.80 × 4.20 to 5.60 µm (23.80 µm × 4.90 µm).

Colonies of *M. desmazierii* on MEA were lax and fluffy, circular in form and terminated at the edges of the plate (Supplementary Fig. 1C). The colonies had a flat surface, with white-yellow mycelium and conidia were rarely observed. On FGPNA, colonies grew similar to MEA with the exception that the mycelium remained white and conidia were frequently observed. Conidia were produced as white-buff conidial masses. The conidia were oblong, hyaline, with rounded edges and smooth walls, measuring 5.90 to 12.20 × 0.90 to 1.50 µm (8.40 µm × 1.20 µm) (Supplementary Fig. 1G).



Supplementary Fig. 1. Micrographs of *Meloderma desmazierii* collected from *Pinus radiata* in Chile. **A,** Ascomata on needle surface. **B,** Close-up of a single ascocarp. **C,** Culture morphology on MEA. **D,** Midpoint vertical section shows the epidermal positioning of two ascocarps with displaced epidermal cells at the base (arrow). **E,** Asci. **F,** Ascospores. **G,** Conidia. Scale bars: B = 500um; D = 100um; E = 10um; F = 20um; G = 10um.

SUMMARY

Commercial plantations of non-native *Pinus* spp. have been established in the Southern Hemisphere for almost two centuries and these plantations have contributed substantially to world trade in wood and wood products. Several different *Pinus* spp. were introduced for site selection and breeding, and this led to increasing movement of plant material across continents. Consequently, there have been several reports of emerging foliar diseases of *Pinus* spp. in the Southern Hemisphere, where the pathogens have most likely been accidentally introduced along with the planting material.

The first chapter of this dissertation provides a review of eight diseases that have had a negative impact on commercial plantations in the Southern Hemisphere or that might pose a risk in the future. A few of these species causing disease have been extensively studied and, with the advent of DNA sequencing and phylogenetic techniques to identify microorganisms, it is evident that several of these include cryptic taxa. This is especially important given that, of the 96 reports of disease outbreaks reported on non-native *Pinus* spp. in the Southern Hemisphere, the causal agent has been confirmed using molecular techniques in only 35 cases. This review, therefore, highlights a serious need to validate historical reports and thus to determine the pathways of movement of pathogens and whether any new invasions may have occurred.

The research presented in the second chapter of the dissertation has recently been published in the journal *Plant Disease*, where the aim was to determine the causal agent of a recent outbreak of Brown spot needle blight on non-native Mesoamerican *Pinus* spp. commercially grown in Colombia. Based on morphological observations and multigene phylogenetic analyses, the causal agent was identified as *Lecanosticta pharomachri*, a species recently described from Guatemala and Honduras. This is the first reports of the species outside its apparent native range and occurring on the hosts *Pinus patula* and *Pinus maximinoi.* This study provided the opportunity to examine the disease symptoms, caused by this species, directly from infected needles and to characterize the sexual state, which was included in an amended species description.

Variability observed in the sequence data of the population of *L. pharomachri* in Colombia suggests that multiple introductions of this pathogen have occurred into that country in the past, most likely with planting material. Interestingly, none of the isolates obtained from a wide area across Colombia were identified as *L. acicola*, which was previously reported in Colombia, and again emphasizes the importance of using DNA sequence data to accurately identify species of *Lecanosticta*.

The third chapter of the dissertation focussed on identifying isolates of *Lophodermium* spp. obtained from non-native *Pinus* spp. in the Southern Hemisphere using DNA sequence and morphological data. These isolates were obtained either directly from the ascocarps of infected needles collected from Australia, Chile, Colombia and South Africa or from historical samples preserved in New Zealand and South African culture collections. Phylogenetic analyses of multiple gene regions revealed the presence of five *Lophodermium* species previously reported in the Southern Hemisphere. Isolates belonging to the *L. conigenum-australe* complex were collected from all countries, except Chile. Isolates belonging to *L. indianum* and *L. molitoris* were found only in Colombian and New Zealand collections, respectively. Within *L. pinastri,* two well supported lineages were observed that may represent cryptic species and which were designated *L. pinastri* A and *L. pinastri* B in this study. These lineages were collected from Chile, New Zealand and Australia. This illustrates that several independent introductions of these fungi have occurred into the Southern Hemisphere from the Northern Hemisphere in the past, most likely with seed and plant material. Several new hosts were discovered for the different *Lophodermium* spp., mainly on tree species native to Mesoamerica. The work highlighted that the morphological features traditionally used for identification of *Lophodermium* spp. are highly variable, especially when infecting different host species. Even though DNA sequence data was useful in distinguishing between the different taxa*,* it was evident that future studies will need to focus on the recollection and typification of *Lophodermium* spp. to provide a resolved phylogeny that will allow for accurate species identification

The findings arising from the studies presented in this dissertation illustrate how anthropogenic activities have led to the introduction and distribution of microbial organisms, including pathogens, into non-native *Pinus* spp. commercially grown in the Southern Hemisphere. They have also highlighted the importance of using DNA

sequence data in conjunction with the morphological observations to accurately identify the fungal species, especially for species from the genera *Lecanosticta* and *Lophodermium.* The studies have also identified several new pine hosts for the fungal species studied, especially *Pinus* spp. native to Mesoamerica. Extreme caution thus should be applied to prevent the accidental introduction of these fungal species together with plant material in the future . This is especially as Mesoamerican *Pinus* spp. are becoming increasingly important to the commercial forestry industry in the Southern Hemisphere.