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ORIGINAL ARTICLE

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

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Abstract

Lophodermium species are amongst the most commonly isolated endophytic fungi on the needles and cones of pines. Of the 38 species reported on these trees, only *Lophodermium seditiosum* is considered a major pathogen. Species of *Pinus* have been widely established as non-natives in Southern Hemisphere countries, and several *Lophodermium* species have been reported on the needles of these trees. However, most of these reports are based on morphology alone. In this study, we considered the biogeography of *Lophodermium* species across the Southern Hemisphere by obtaining and identifying isolates from non-native *Pinus* species planted in Australia, Chile, Colombia, New Zealand, and South Africa. A multi-locus phylogenetic approach was used to delineate the species, and characteristic morphological features were evaluated against the resulting phylogeny. Phylogenetic analyses revealed the presence of five *Lophodermium* taxa on *Pinus* species in the Southern Hemisphere. A species belonging to the *L. conigenum-australe* complex was found in all countries except Chile. *Lophodermium indianum* and *L. molitoris* were found only in Colombian and New Zealand collections, respectively. Two distinct lineages of *L. pinastri* emerged from Chile, New Zealand, and Australia. None of the morphological features could distinguish between the different taxa of *Lophodermium* found, with several of the traits varying by host or location. Overall, the results support the fact that various cryptic *Lophodermium* species occur on the sampled *Pinus* species and suggest that several independent introductions of these fungi have occurred in Southern Hemisphere countries.

Keywords Biosecurity · Endophyte · Invasive · *Leotiomycetes* · Pine needle diseases · Phylogeny

Introduction

Endophytic fungi of plants are mainly horizontally transferred, and the community composition is highly dependent on host and geographic location (Christian et al. 2016). In conifers, where needles are retained for long periods, endophytic communities are relatively diverse (Sieber 2007). Studies of fungal endophytes infecting needles of *Pinus* L. species native to the Northern Hemisphere have shown that members of *Rhytismataceae* are dominant (Carroll and Carroll 1978; Hata and Futai 1996; Ortiz-García et al. 2003; Ganley et al. 2004). These include species of *Lophodermium* Chevall., for which approximately 38 species have been described from needles and cones (Salas-Lizana and Oono 2018a).

The majority of the *Lophodermium* species 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 affecting

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trees in nurseries and plantations (Minter and Millar 1980; Staley and Nicholls 1989; Jansons et al. 2020). This species causes needle cast that can result in a reduction in tree height and diameter and occasionally seedling death in nurseries.

Lophodermium species have been described based on the internal and external morphological features of the ascomata on pine 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 anatomy of the *Pinus* species being colonised (Minter 1981; Johnston et al. 2003; Ortiz-García et al. 2003).

The first comprehensive phylogenetic study on *Lophodermium* species utilised 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 for 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) also highlighted the similarities in the ascomata of *Lophodermium conigenum* (Brunaud) Hilitzer and *Lophodermium australe* Dearn. Subsequently, phylogenetic analyses using sequence data for the ITS region suggested that these species may be conspecific (Prihatini et al. 2016; Salas-Lizana and Oono 2018b).

Several recent studies have considered the endophytic communities of native and planted *Pinus* species in the Northern Hemisphere. They utilised molecular genetic data and revealed 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 2018a). These studies used the partial actin (*ACT*) gene region as a secondary marker, but this did not provide better resolution than the ITS region for the *Lophodermium* species considered (Koukol et al. 2015; Tanney and Seifert 2017; Salas-Lizana and Oono 2018a). The translation elongation factor 1-α (*TEF*1) gene was identified as a promising phylogenetic marker, and it has been suggested that it could be useful in future phylogenetic studies of the *Rhytismatales* (Stielow et al. 2015; Tanney and Seifert 2017).

There are approximately 120 species of *Pinus* known globally, and all but one species, *Pinus merkusii* Jungh. & de Vriese, occur naturally in the Northern Hemisphere (Cooling 1968; Richardson 1998). Several species were introduced into the Southern Hemisphere as ornamentals, and plantations of *Pinus* were established in various countries from the late nineteenth century. Concurrent with this development, numerous pathogens appeared on these trees, including those that had been inadvertently introduced along with planting material (Gibson 1979; Wingfield 1999; Burgess and Wingfield 2001). One of the earliest reports of a species of *Lophodermium* in the Southern Hemisphere was of *Hysterium pinastri* (now *L. pinastri*) suggested to be causing tip dieback on *Pinus radiata* D. Don and *Pinus pinea* L. in South Africa (Zahn and Neethling 1929; Lundquist 1987) and 'pine needle split' on *Pinus* species in Australia (Nahrung and Carnegie 2020). Since then, several other species of *Lophodermium* have been reported on *Pinus* species in Southern Hemisphere countries (Roux and Lundquist 1984; Johnston et al. 2003; Prihatini et al. 2016).

Most reports of *Lophodermium* species on non-native *Pinus* species in the Southern Hemisphere have relied solely on morphological characteristics for identification. This has resulted in reports of the pathogen *L. seditiosum* from both Chile and South Africa (Roux and Lundquist 1984; Butin and Peredo 1986) although there has been no evidence of disease outbreaks caused by this fungus. This calls into question the reliability of those, and most likely other, reports of these fungi from Southern Hemisphere countries. Identifications based on DNA sequence data have been performed for only a small number of isolates from *Pinus* species 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).

In this study, we aimed to identify *Lophodermium* species collected from *Pinus* species in Southern Hemisphere countries using DNA sequence data. Due to the availability of existing cultures or the possibility of collecting new samples from well-established *Pinus* plantations, our focus was on Australia, Chile, Colombia, New Zealand, and South Africa. Although Colombia is not strictly in the Southern Hemisphere, collections were included due to the country's position close to the equator and because the non-native *Pinus* planted are similar to those in other Southern Hemisphere countries sampled. The objectives were to (i) obtain the largest possible collection of *Lophodermium* samples from non-native *Pinus* species grown in these five countries, (ii) isolate and confirm the identity of the fungal species using DNA sequence and phylogenetic analyses of the ITS and *ACT* regions, (iii) determine the suitability of the translation elongation factor 1-α gene region (*TEF*1) to resolve the identity of the isolated *Lophodermium* species, and (iv) use the phylogenies to identify any shared and unique morphological characteristics amongst *Lophodermium* species that may aid in delineating species boundaries.

Materials and methods

Sample collection and isolations

Isolates of *Lophodermium* species were obtained directly from infected needles collected in Australia, Chile, Colombia, and South Africa or from isolates preserved in New Zealand and South African culture collections. Needles bearing ascomata resembling *Lophodermium* were collected from trees between February 2020 and March 2021. They were placed in labelled paper bags within larger plastic bags and stored at 4 °C 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 those with characteristic ascomata (Minter 1981) were chosen from different sites and hosts from each country (Table 1). Needles were sprayed with 70% ethanol, wiped clean using paper towels moistened with distilled water, and placed in moist chambers for four hours or overnight. Ascomata from hydrated needles were excised using a sterile scalpel blade and macerated on the surface of 2% malt extract agar (MEA, 20 g/L malt extract, 20 g/L agar; Biolab). The resulting cultures were incubated at 20–25 °C for 24–48 h, after which single germinating spores or hyphal tips were sub-cultured onto 2% MEA to obtain pure cultures.

Herbarium specimens of *Lophodermium* species were obtained from the National Forestry Fungarium (NZFRIM), Scion, New Zealand. Cultures from New Zealand were supplied by the National Forest Culture Collection (NZFS) at Scion. Cultures from Colombia 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 those isolated in this study were grouped based on morphology. All isolates used in this study have been preserved in the CMW culture collection. Representative needle samples for the Australian collections are also stored in the NSW Plant Pathology Herbarium (DAR).

DNA extraction and PCR amplifications

One-week-old cultures were used in DNA extractions by macerating fungal mycelium with a sterile toothpick and using 50 µL Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's recommended protocols. The following primer sets were used to amplify the respective gene regions: ITS1 and ITS4 (White et al. 1990) for the internal transcribed spacer rDNA (ITS), ACT-512F and ACT-783R (Carbone and Kohn 1999) for the partial actin (*ACT*) gene region, and EF-728F and EF-986R (Carbone and Kohn 1999) for the translation elongation factor 1-α (*TEF*1). PCR amplifications were performed in 13 µL reactions containing 2.5 µL 5 × MyTaq buffer (Bioline, London, UK), 0.25 µL MyTaq DNA polymerase (Bioline), 1 µL DNA template (Prepman extraction), 0.5 µL of each primer (10 mM), and 8.25 µL sterile SABAX water. PCR conditions for all reactions were the same as those used by Tanney and Seifert (2017) on an Applied Biosystems Veriti© 96-well Thermal Cycler (Thermo Fisher Scientific, Massachusetts, USA). Amplification of all PCR products was confirmed by staining 2 µL product with 1 µL GelRed™ Nucleic Acid Gel Stain (Biotium, California, 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 ascomata, which were crushed in 30 µL TE Buffer (10 mM Tris, 1 mM EDTA) using a sterilized toothpick and heated at 95 °C for 15 min followed by 80 °C 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 described above.

Sequencing and phylogenetic analyses

PCR products were cleaned in a 6.7% G-50 Sephadex solution (MilliporeSigma, Massachusetts, USA) using Centrisep 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), 2 µL sequencing buffer, 1 µL of either the forward or reverse primer, and 5 µL sterile SABAX water. Products were sequenced on the ABI PRISM™ 3500xl Auto-sequencer (Applied Biosystems, Thermo Fisher Scientific) at the sequencing facility of the University of Pretoria, Pretoria, South Africa. The forward and reverse sequencing reads were imported into CLC Bio Main Workbench v8 (QIAGEN, https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-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 species of *Lophodermium*.

Previously deposited ITS sequences for isolates of *Lophodermium* collected in Colombia and New Zealand were retrieved from GenBank and analysed together with the

Table 1 Information for isolates of *Lophodermium* species collected on *Pinus* species propagated in Southern Hemisphere countries (including Colombia) where these trees do not naturally occur

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* × *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* × *P. caribaea*	M. Ramsden & H. Nahrung	December 2020
L. conigenum-australe complex	Australia	Queensland	CMW58467[d]	Needles	*P. elliottii* × *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

Table 1 (continued)

Taxon	Country	Region	Culture collection number[a,b,c]	Source[f]	Host	Collector	Collection date
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* × *P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58491[d]	Needles	*P. elliottii* × *P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58488[d]	Needles	*P. elliottii* × *P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	KwaZulu-Natal	CMW58489	Needles	*P. elliottii* × *P. caribaea*	M.J. Wingfield	October 2020
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58501	Needles	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58502	Needles	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2021
L. conigenum-australe complex	South Africa	Mpumalanga	CMW58503	Needles	*P. elliottii* × *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* × *P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54461[d]	Culture	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54462	Culture	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54463[e]	Culture	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2019

Table 1 (continued)

Taxon	Country	Region	Culture collection number[a,b,c]	Source[f]	Host	Collector	Collection date
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54464[d]	Culture	*P. elliottii* × *P. caribaea*	M.J. Wingfield	April 2019
L. conigenum-australe complex	South Africa	Mpumalanga	CMW54465[d]	Culture	*P. elliottii* × *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* × *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 indianum	Colombia	Cundinamarca Department	CMW55979[d]	Culture	*P. patula* × *P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55980[d]	Culture	*P. patula* × *P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55981	Culture	*P. patula* × *P. tecunumanii*	M.J. Wingfield	February 2020
L. indianum	Colombia	Cundinamarca Department	CMW55982[d]	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

Table 1 (continued)

Taxon	Country	Region	Culture collection number[a,b,c]	Source[f]	Host	Collector	Collection date
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*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW58484	Needles	*P. maximinoi*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW58485[d]	Needles	*P. patula* × *P. tecunumanii*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Risaralda Department	CMW56845[d]	Needles	*P. patula* × *P. tecunumanii*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56846[e]	Needles	*P. maximinoi*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56847[e]	Needles	*P. tecunumanii*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58481[e]	Needles	*P. tecunumanii*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW56848	Needles	*P. maximinoi*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58482	Needles	*P. maximinoi*	C.A. Rodas & M.D. Bolaños	February 2020
L. indianum	Colombia	Valle del Cauca Department	CMW58483	Needles	*P. maximinoi*	C.A. Rodas & 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
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

Table 1 (continued)

Taxon	Country	Region	Culture collection number[a,b,c]	Source[f]	Host	Collector	Collection date
L. pinastri	Chile	Los Ríos	CMW58476[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	February 2021
L. pinastri	New Zealand	Dunedin	NZFRI-M3975[d]	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[d]	Needles	*P. radiata*	R. Ahumada & J. Aguayo	January 2021

Samples from Australia are logged in the NSW Plant Pathology and Mycology Herbarium (DAR)

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

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

[c]*NZFRIM* National Forest Fungarium, 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 to obtain an identification

sequences generated in this study (Table S1). Sequences for ITS and *ACT* with similar species identity were aligned in CLC Bio Main Workbench v8, 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 (Table S1) was chosen for collections from all five 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 species of *Lophodermium*. ITS, *ACT* and *TEF*1 sequences for closely related taxa from different countries were retrieved from public databases (Table S2), with a focus on the species known to be associated with species of *Pinus*. 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* (Pers.) Wallr. and *Tryblidiopsis pinastri* (Pers.) P. Karst. were used as the outgroup taxa for the ITS phylogeny (Koukol et al. 2015; Bartnik et al. 2021) and *Hymenoscyphus epiphyllus* (Pers.) Rehm ex Kauffman 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* (Berk. & Broome) Höhn. was used as the outgroup taxon following its placement relative to *Lophodermium* species in a previous study by Prihatini et al. (2014). Phylogenetic trees were visualized in FigTree v1.4.4 and edited in Affinity Designer v1.10.4.

Morphological observations

Morphological characteristics of the ascomata were studied using structures taken directly from the needles of representative specimens. These specimens were chosen based on the *Lophodermium* clades resulting from the phylogenetic analyses (Table 1). This included specimens from each of the sampled countries and different *Pinus* species. 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 dissecting microscope and photographed using a ZEISS Camera AxioCam ICc5 (Carl Zeiss AG, Oberkochen Germany). To observe the position of the ascomata 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, Illinois, USA). Transverse Sects. (50 µm thickness) were cut through the fruiting structures using a freezing microtome (Leica Biosystems). The sections, including asci and ascospores, were mounted in 85% lactic acid and examined using a Nikon H550L microscope (Nikon, Yokohama, Japan) with images captured using a Nikon DS-Ri2 camera.

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

Results

Sample collection and isolations

A total of 99 isolates tentatively identified as species of *Lophodermium* were obtained either from isolations made from ascomata on needles of *Pinus* spp. in this study or previously preserved cultures obtained from culture collections (Table 1). These included 15 isolates from Australia, 12 from Chile, 27 from Colombia, 18 from New Zealand and 29 from South Africa.

Sequencing and sequence analyses

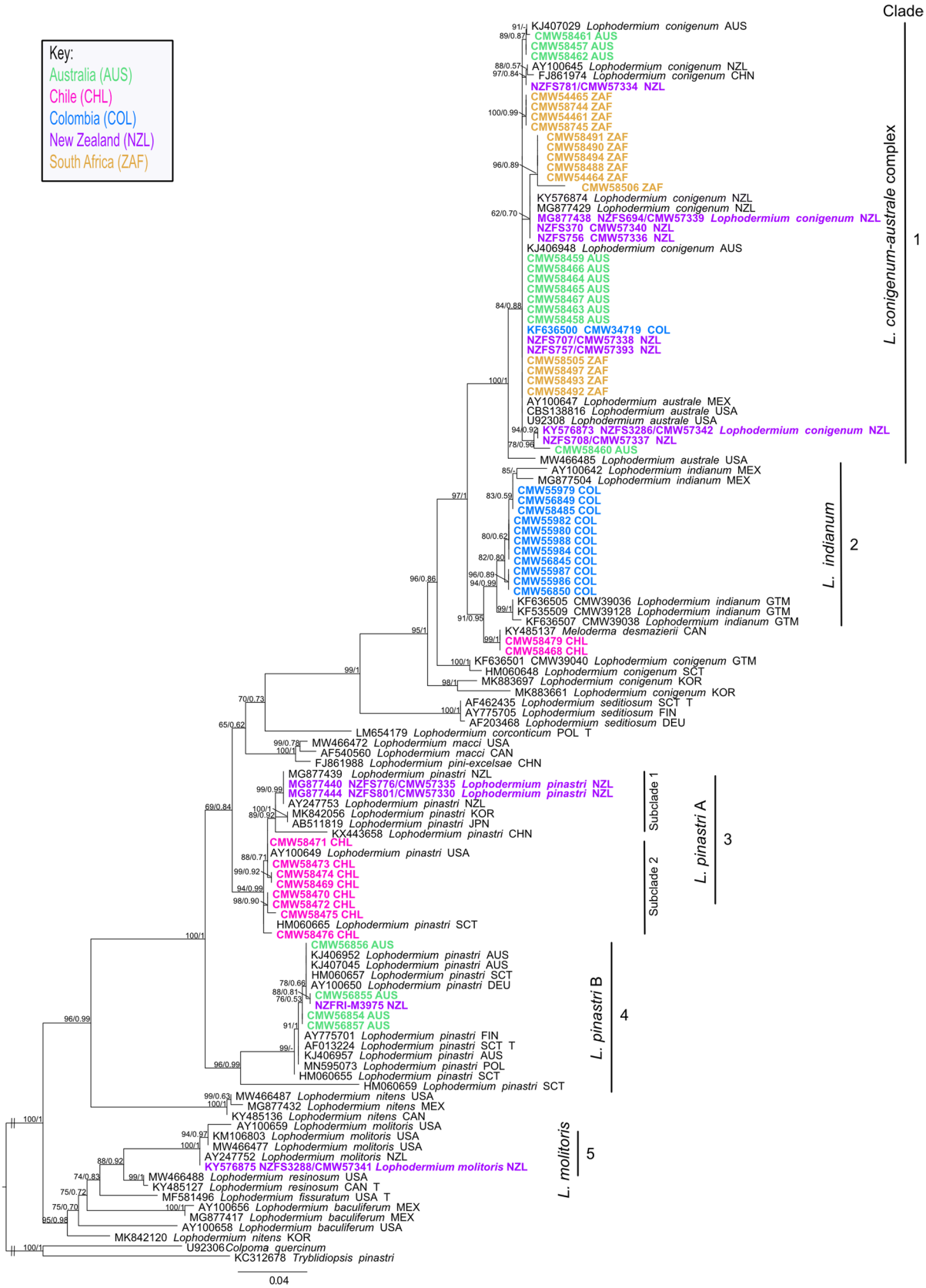
The ITS region was successfully amplified and sequenced for 92 isolates originating from the five countries considered. In addition, sequences for the seven remaining isolates from New Zealand characterized in a previous study (Salas-Lizana and Oono 2018a, b) were retrieved from GenBank. Two ITS amplicons were successfully generated from a direct PCR of two individual ascoma from the fungarium specimens NZFRI-M3975 and NZFRI-M4654 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 species of *Lophodermium*. The 94 ITS sequences generated were added to the sequences retrieved from GenBank, and alignments of the 101 sequences revealed a total of 38 unique ITS haplotypes (Table S1). A subset of 63 ITS sequences representing the 38 unique haplotypes for isolates from the five sampled countries and those from different *Pinus* species was used for the phylogenetic analysis.

The *ACT* region was successfully amplified for 97 isolates (Table S1). The average fragment length of the sequences was 267 bp, and a total of 41 unique *ACT* haplotypes were observed (Table S1). A subset of 62 *ACT* sequences representing the unique *ACT* haplotypes was used for the phylogenetic analysis.

The *TEF*1 gene region was amplified for 55 representative isolates based on the ITS and *ACT* haplotypes obtained, and the average fragment length was 240 bp. Sequence alignments revealed 28 unique *TEF*1 haplotypes, and all 55 sequences were used in the phylogenetic analyses (Table S1). All sequences generated in this study were deposited in GenBank (Table S1).

Phylogenetic analyses

High levels of nucleotide variability were observed in the sequences across all three gene regions for the


Key:
Australia (AUS)
Chile (CHL)
Colombia (COL)
New Zealand (NZL)
South Africa (ZAF)
Clade
KJ407029 Lophodermium conigenum AUS
CMW58461 AUS
CMW58457 AUS
CMW58462 AUS
AY100645 Lophodermium conigenum NZL
FJ861974 Lophodermium conigenum CHN
NZFS781/CMW57334 NZL
CMW54465 ZAF
CMW58744 ZAF
CMW54461 ZAF
CMW58745 ZAF
CMW58491 ZAF
CMW58490 ZAF
CMW58494 ZAF
CMW58488 ZAF
CMW54464 ZAF
CMW58506 ZAF
KY576874 Lophodermium conigenum NZL
MG877429 Lophodermium conigenum NZL
MG877438 NZFS694/CMW57339 Lophodermium conigenum NZL
NZFS370 CMW57340 NZL
NZFS756 CMW57336 NZL
KJ406948 Lophodermium conigenum AUS
CMW58459 AUS
CMW58466 AUS
CMW58464 AUS
CMW58465 AUS
CMW58467 AUS
CMW58463 AUS
CMW58458 AUS
KF636500 CMW34719 COL
NZFS707/CMW57338 NZL
NZFS757/CMW57393 NZL
CMW58505 ZAF
CMW58497 ZAF
CMW58493 ZAF
CMW58492 ZAF
AY100647 Lophodermium australe MEX
CBS138816 Lophodermium australe USA
U92308 Lophodermium australe USA
KY576873 NZFS3286/CMW57342 Lophodermium conigenum NZL
NZFS708/CMW57337 NZL
CMW58460 AUS
MW466485 Lophodermium australe USA
AY100642 Lophodermium indianum MEX
MG877504 Lophodermium indianum MEX
CMW55979 COL
CMW56849 COL
CMW58485 COL
CMW55982 COL
CMW55980 COL
CMW55988 COL
CMW55984 COL
CMW56845 COL
CMW55987 COL
CMW55986 COL
CMW56850 COL
KF636505 CMW39036 Lophodermium indianum GTM
KF535509 CMW39128 Lophodermium indianum GTM
KF636507 CMW39038 Lophodermium indianum GTM
KY485137 Meloderma desmazierii CAN
CMW58479 CHL
CMW58468 CHL
KF636501 CMW39040 Lophodermium conigenum GTM
HM060648 Lophodermium conigenum SCT
MK883697 Lophodermium conigenum KOR
MK883661 Lophodermium conigenum KOR
AF462435 Lophodermium seditiosum SCT T
AY775705 Lophodermium seditiosum FIN
AF203468 Lophodermium seditiosum DEU
LM654179 Lophodermium corconticum POL T
MW466472 Lophodermium macci USA
AF540560 Lophodermium macci CAN
FJ861988 Lophodermium pini-excelsae CHN
MG877439 Lophodermium pinastri NZL
MG877440 NZFS776/CMW57335 Lophodermium pinastri NZL
MG877444 NZFS801/CMW57330 Lophodermium pinastri NZL
AY247753 Lophodermium pinastri NZL
MK842056 Lophodermium pinastri KOR
AB511819 Lophodermium pinastri JPN
KX443658 Lophodermium pinastri CHN
CMW58471 CHL
AY100649 Lophodermium pinastri USA
CMW58473 CHL
CMW58474 CHL
CMW58469 CHL
CMW58470 CHL
CMW58472 CHL
CMW58475 CHL
HM060665 Lophodermium pinastri SCT
CMW58476 CHL
CMW56856 AUS
KJ406952 Lophodermium pinastri AUS
KJ407045 Lophodermium pinastri AUS
HM060657 Lophodermium pinastri SCT
AY100650 Lophodermium pinastri DEU
CMW56855 AUS
NZFRI-M3975 NZL
CMW56854 AUS
CMW56857 AUS
AY775701 Lophodermium pinastri FIN
AF013224 Lophodermium pinastri SCT T
KJ406957 Lophodermium pinastri AUS
MN595073 Lophodermium pinastri POL
HM060655 Lophodermium pinastri SCT
HM060659 Lophodermium pinastri SCT
MW466487 Lophodermium nitens USA
MG877432 Lophodermium nitens MEX
KY485136 Lophodermium nitens CAN
AY100659 Lophodermium molitoris USA
KM106803 Lophodermium molitoris USA
MW466477 Lophodermium molitoris USA
AY247752 Lophodermium molitoris NZL
KY576875 NZFS3288/CMW57341 Lophodermium molitoris NZL
MW466488 Lophodermium resinosum USA
KY485127 Lophodermium resinosum CAN T
MF581496 Lophodermium fissuratum USA T
AY100656 Lophodermium baculiferum MEX
MG877417 Lophodermium baculiferum MEX
AY100658 Lophodermium baculiferum USA
MK842120 Lophodermium nitens KOR
U92306 Colpoma quercinum
KC312678 Tryblidiopsis pinastri
0.04
L. conigenum-australe complex
1
L. indianum
2
Subclade 1
Subclade 2
L. pinastri A
3
L. pinastri B
4
L. molitoris
5

◂**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 the key, fall within five clades of recognized *Lophodermium* species. Ex-type sequences are indicated with a "T" and *Colpoma quercinum* and *Tryblidiopsis pinastri* were used as the outgroup taxa. Numbers at the nodes indicate the bootstrap support (> 60%) and the posterior probabilities from Bayesian inference, respectively

Lophodermium species. Sequences for ex-type specimens of species in the *Rhytismatales* were underrepresented, and a lack of sequences available for the *ACT* and *TEF*1 regions precluded a combined analysis. Consequently, individual datasets were used. The ITS dataset consisted of 123 sequences with 570 aligned characters and 157 parsimony-informative sites. The *ACT* dataset consisted of 80 sequences with 307 aligned characters and 137 parsimony-informative sites. The *TEF*1 dataset comprised 62 sequences with 268 aligned characters and 153 parsimony-informative sites. According to the Bayesian information criterion (BIC), the best-fit substitution model was TIM2e + I + G4 for ITS and *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.

Phylogenetic analyses for the ITS (Fig. 1), *ACT* (Fig. S1) and *TEF*1 (Fig. S2) regions showed that there were five strongly supported clades of *Lophodermium* present on non-native *Pinus* species considered in this study (Fig. 2). These clades were closely aligned with *Lophodermium* species previously characterized for these gene regions. There were no new records of *Lophodermium* species for the countries considered, and apart from the *L. conigenum-australe* complex, there was little overlap in the species present across all countries considered. The phylogenetic clades in Figures S1 and S2 are labelled to be consistent with the clades assigned in Fig. 1 based on analysis of the ITS region.

Clades and species identified

Clade 1: *Lophodermium conigenum-australe* complex

Most of the sequences fell within clade 1, which accommodated isolates collected from ten different *Pinus* hosts in Australia, Colombia, New Zealand, and South Africa. The ITS analyses revealed nineteen unique ITS haplotypes (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 value of 84% (Fig. 1). The *ACT* analyses revealed 20 unique *ACT* haplotypes (Table S1), where the isolates grouped with two sequences available for *L. australe* from the USA with a bootstrap support of 99% (Fig. S1). This clade was also resolved in the *TEF*1 analysis (Fig. S2), revealing 11 unique haplotypes (Table S1).

Clade 2: *Lophodermium indianum*

Clade 2 accommodated only sequences from Colombia collected from Mesoamerican *Pinus* species and their hybrids (*P. maximinoi* H.E. Moore, *P. tecunumanii* Eguiluz & J. P. Perry., *P. patula* Schiede ex Schltdl. & Cham., *P. patula* × *P. tecunumanii*). This clade included seven distinct ITS haplotypes (Table S1) that grouped with sequences previously identified as *L. indianum* Suj. Singh & Minter from Mexico with a bootstrap support of 94% (Fig. 1). These sequences were also closely related to those of *L. indianum* from *P. maximinoi* in Guatemala.

The *ACT* analysis revealed six unique haplotypes (Table S1). However, the isolates from Colombia formed a polytomy (Fig. S1). Furthermore, in the *TEF*1 analysis, several Colombian isolates were paraphyletic to the rest of the Colombian isolates (Fig. S2). Therefore, all isolates residing in clade 2 were treated as *L. indianum.*

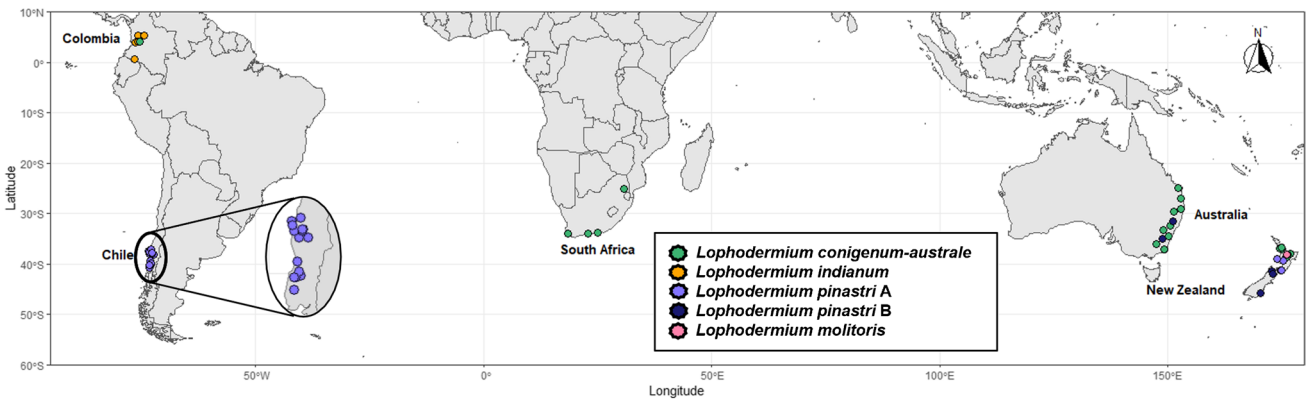


Fig. 2 Distribution of *Lophodermium* species on non-native *Pinus* species from Australia, Chile, Colombia, New Zealand, and South Africa

The sequences for two isolates obtained from *P. radiata* from Chile grouped close to, but separate from, clade 2 in all the gene regions analysed (Fig. 1, Fig. S1 and Fig. S2). These isolates were tentatively identified as *Meloderma desmazierii* (Duby) Darker with a percentage identity of 100% based on the NCBI BLASTn analysis of the ITS sequence (Accession No. KY485137). In the ITS analysis, these sequences represented a single haplotype and grouped with an isolate of *M. desmazierii* from Canada. However, no additional sequence data for the other gene regions were available for this species, and in the *ACT* and *TEF*1 analyses, this haplotype formed its own clade.

Clade 3: *Lophodermium pinastri* A

The ITS, *ACT*, and *TEF*1 analyses grouped the isolates residing in clade 3 into two subclades. Subclade 1 accommodated isolates from New Zealand collected from five different *Pinus* species. For the ITS analyses, these isolates represented a single ITS haplotype (Table S1) that grouped with sequences of *L. pinastri* previously identified from New Zealand and that are closely related to isolates of *L. pinastri* from East Asia (Fig. 1). The *ACT* (Fig. S1) and *TEF*1 (Fig. S2) analyses revealed two different haplotypes in these isolates (Table S1) which grouped with isolates of *L. pinastri* from Scotland and New Zealand. Subclade 2 included 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 (Table S1). In the ITS (Fig. 1) and *ACT* (Fig. S1) analyses, these isolates grouped with sequences of *L. pinastri* from Scotland. In the *TEF*1 analysis, the sequences for isolates from Chile resulted in five haplotypes (Table S1) that formed two lineages (Fig. S2). Until additional sequence data become available to resolve the subclades possibly as different species, all isolates residing in this clade were identified as *L. pinastri* A.

Clade 4: *Lophodermium pinastri* B

Clade 4 accommodated sequences for isolates from five *Pinus* species from Australia and New Zealand. The ITS analysis revealed three different ITS haplotypes (Table S1), two haplotypes present only in Australian collections 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 a previous study (Prihatini et al. 2016). Other sequences of *L. pinastri* from countries such as Finland, Poland, Germany, Scotland, and Australia were also present in this clade (Fig. 1). Analyses of the *ACT* sequences revealed four unique haplotypes (Table S1) for the isolates from Australia that grouped with sequences of *L. pinastri* from Scotland with a bootstrap support of 99% (Fig. S1). Similarly, the Australian isolates residing in clade 4 included three haplotypes in the *TEF*1 analyses (Table S1) that were also well supported in the tree and closely related to *L. pinastri* previously collected in Australia (Fig. S2). Isolates residing in this clade were identified as *L. pinastri* B.

Clade 5: *Lophodermium molitoris*

A single isolate of *L. molitoris* from *P. radiata* collected in 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 the USA with 100% bootstrap (Fig. 1). For the *ACT* (Fig. S1) and *TEF*1 (Fig. S2) analyses, the isolate grouped with sequences from the USA and New Zealand, respectively, with a high bootstrap support.

Morphological observations

The phylogenetic analyses of the isolates considered in this study provided a basis to determine whether morphological features could be linked to a specific clade. Very few morphological traits were distinct for the clades examined, with overlap in several of the features across the different clades (Table 2). Clade 1, representing the *Lophodermium conigenum-australe* complex, showed the highest level of variation in most features, depending on the host and location from which the material had originated (Fig. 3). Variation was also observed in the culture morphology for isolates in this clade and those belonging to Clade 2, representing *L. indianum* (Fig. 4). However, isolates in Clade 2 were distinct because they did not produce conidia in culture.

Extensive overlap was observed in the features of *L. pinastri* A (Clade 3) and *L. pinastri* B (Clade 4), but they produced black zone lines on the needles which made them distinct from collections representing other clades (Table 2). Two different culture morphologies were observed for the isolates in *L. pinastri* A belonging to either subclade 1 or subclade 2 (Fig. 5). However, the culture characteristics of isolates in clade 4 on MEA were similar to those of *L. pinastri* A sub-clade 2. Red clypeal 'lips' were observed for a single individual from clade 4 (Fig. 6). Cultures of isolates belonging to clade 5, representing *L. molitoris,* resembled those in the *L. conigenum-australe* complex (Fig. 7) and their conidia were also similar in size (Table 2).

Meloderma desmazierii, an endophyte first found on *P. strobus* L. in Canada, could be distinguished from species of *Lophodermium* by the ITS, *ACT* and *TEF*1 analyses. However, it fell within the same lineage as *L. indianum*.

Table 2 Comparison of ascocarp and culture morphology in *Lophodermium* collections residing in the five phylogenetic clades resolved in this study

Features	Clade 1. *Lophodermium conigenum-australe* complex	Clade 2. *Lophodermium indianum*	Clade 3. *Lophodermium pinastri* A[a]	Clade 4. *Lophodermium pinastri* B	Clade 5. *Lophodermium molitoris*[b]
Ascomata external features					
Shape	Thin, elongated, or elliptical	Elliptical	Oval to elliptical	Oval to elliptical	—
Colour	Black	Black	Black	Black	—
Ascocarp surround	Concolorous to needle surface or lighter	Absent or dark grey	Grey	Grey or black	—
Perimeter line	Absent, broken, or solid black Pointed ends	Absent or solid black Pointed ends	Black Rounded ends	Black Rounded ends	—
Size (µm) Length range [average] ×width range [average]	289–491 [366] × 58–85 [74] 494–936 [721] × 75–236 [136]/ 796–1498 [1135] × 117–405 [264]	721–1499 [1092] × 178–449 [304]	367–700 [511] × 158–284 [226]/ 728–826 [770] × 343–442 [399]	632–954 [836] × 275–433 [377]	—
Slit colour of the clypeus	Absent or grey	Grey or black	Grey or black	Grey or black, infrequently red	—
Zone lines	Absent	Absent	Black, frequent	Black, frequent	—
Ascomata internal features					
Ascomata position	Sub-hypodermal or sub-epidermal	Sub-hypodermal	Sub-epidermal	Sub-epidermal	—
Hypodermal cells displaced	Yes	Yes	No	No	—
Number and arrangement of epidermal cells displaced	2, grouped or scattered	0–2, scattered	5–8, grouped	> 4, grouped	—
Ascus (µm) Length range [average] ×width range [average]	40.9–72.7 [55.4] × 5.3–8.4 [6.9]/ 92.2–126.1 [109.1] × 9.4–11.8 [10.8]	37.2–69.4 [54.2] × 5.4–7.8 [6.7]	73.3–123.3 [97.4] × 7.1–12.9 [10.3]	84.7–113.1 [95.3] × 7.0–10.3 [8.9]/ 113.5–142.0 [123.0] × 7.4–11.5 [10.1]	—
Ascospores (µm) Length range [average] ×width range [average]	30.4–43.7 [39.6] × 1–7.8 [1.5]/ 70.6–85.6 [76.9] × 2–3.8 [2.8]	29.6–41.2 [36.2] × 1.1–2.0 [1.5]	31.0–47.7 [38.7] × 1.0–2.1 [1.5]	45.8–62.8 [54.9] × 1.7–1.9 [1.8]/ 69.6–85.2 [77.4] × 1.2–2.5 [1.8]	—
Culture characteristics					
Mycelial growth (MEA)	Fast-growing, circular, and filiform margins terminating before the edges of the plate	Fast-growing, circular in shape and filiform margins, or slow-growing, compact and lobate margins. Terminating early or at the edge of the plate	Subclade 1: slow-growing, compact, filiform margins, umbonate surface and terminated growth early Subclade 2: fast-growing, compact, entire margins, circular in shape, flat surface and terminated at the edges of the plate	Fast-growing, compact, circular in shape, entire margins and terminated at the edges of the plate	Fast-growing, fluffy, circular in shape, filiform margins terminated at the edges of the plate

Table 2 (continued)

Features	Clade 1. *Lophodermium conigenum-australe* complex	Clade 2. *Lophodermium indianum*	Clade 3. *Lophodermium pinastri* A[a]	Clade 4. *Lophodermium pinastri* B	Clade 5. *Lophodermium molitoris*[b]
Culture colour and discolouration (MEA)	White, no discolouration in the agar or tan to brown with a slight brown discolouration in the agar	White to brown or tan, some cultures with tan centre and white edges. Few colonies were buff colour and sepia brown discolouration in agar	Subclade 1: white with patches of salmon to tan Subclade 2: grey-white No discolouration in the agar	Straw-white, occasionally produced black zone line at circumference, no discolouration in the agar	Brown No discolouration in the agar
Culture colour and conidial masses (FGPNA)	White Black conidial masses	White No conidial masses observed	White White–grey conidial masses. Subclade 1 occasionally produced black zone lines at circumference	White White–grey conidial masses	White Black conidial masses
Conidia size (µm) Length range [average] × width range [average]	3.5–4.6 [3.8] × 0.6–0.8 [0.7]/ 2.9–7.4 [5.3] × 0.5–1 [0.7]/ 6.5–8.5 [7.2] × 1–1.4 [1.2]	Not observed	3.8–4.8 [3.9] × 0.5–1.0 [0.7]	3.8–6.8 [5.0] × 1.0–1.6 [1.3]	3.8–4.8 [3.9] × 0.5–1.0 [0.7]

[a]Pine needles used to study the features of the ascomata of *L. pinastri* A were only available for the samples from Chile on *P. radiata*, whilst all samples of this species analysed from New Zealand were in the form of cultures

[b]Only cultures were available for this species in this study

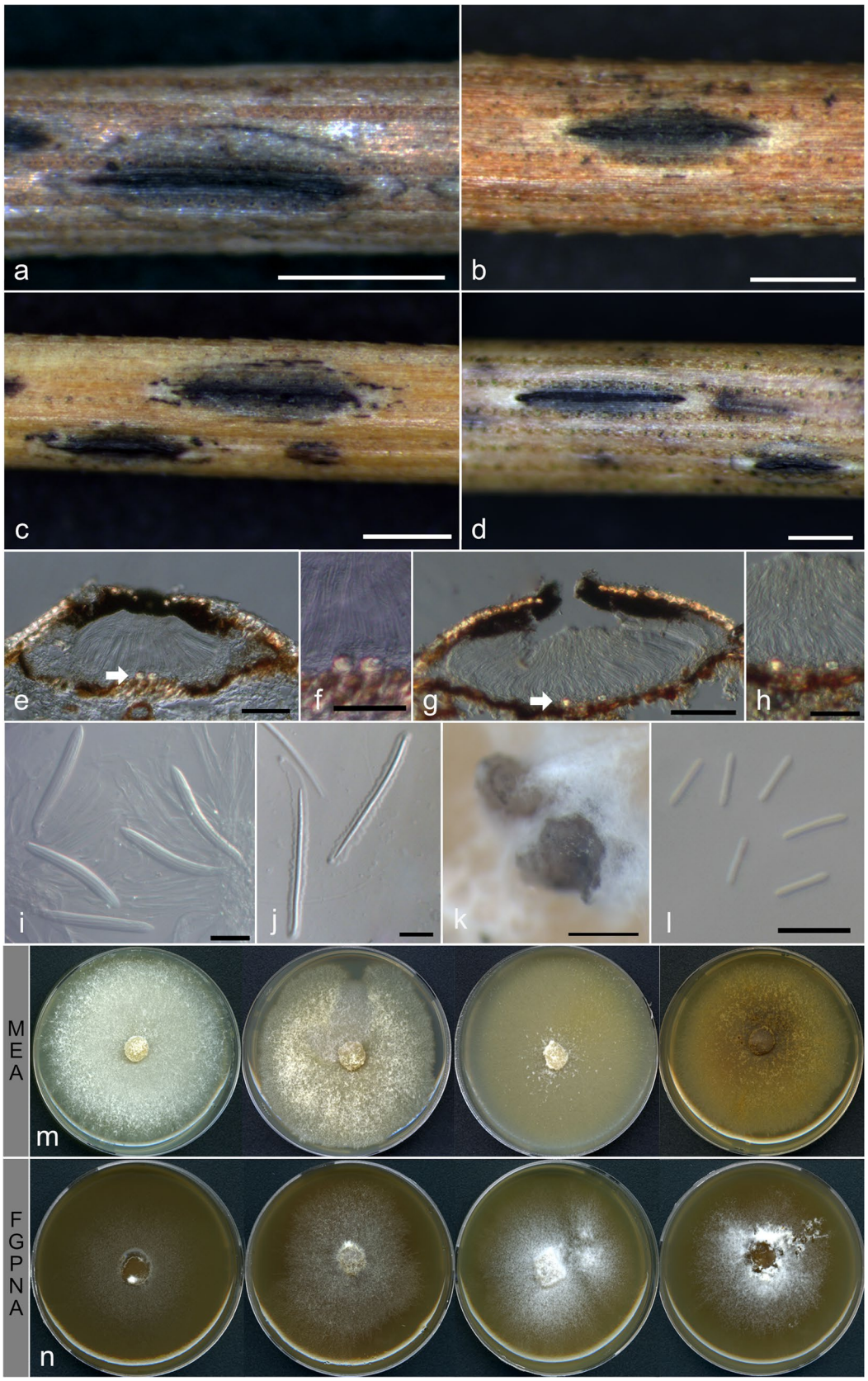
Morphologically, this species resembled the *L. conigenum-australe* complex but was distinct in having shorter ascospores and larger conidia than species of *Lophodermium* considered in the present study (Fig. S3).

Discussion

Multigene phylogenetic analyses based on three gene regions revealed the presence of five well-supported *Lophodermium* taxa on non-native *Pinus* species grown in five countries where these trees have been established in plantations. Two of these species could be identified, with confidence, as *L. indianum* and *L. molitoris* from Colombia and New Zealand, respectively. The remaining three species had taxonomic boundaries that were less clear and were identified as members of the *Lophodermium conigenum-australe* complex or in clades identified as *Lophodermium pinastri* A and *Lophodermium pinastri* B. There was considerable variability in the morphological characters of these fungi, making it difficult to link them conclusively to the species identified based on DNA-sequence data. The study also showed that there have been independent, accidental introductions of *Lophodermium* species into the Southern Hemisphere countries considered, most likely with germplasm used to establish these plantations.

Isolates residing in the *L. conigenum-australe* complex were collected from Australia, Colombia, South Africa, and New Zealand. This complex was confirmed, for the first time based on DNA sequence data, from Colombia and South Africa, occurring on various *Pinus* species. This species, previously identified based only on morphology, had been reported from Chile on several *Pinus* species (Butin and Peredo 1986). Interestingly, it was not found on any of the samples collected from *P. radiata* in Chile and considered in the present study. The results highlight the fact that morphological characteristics used in the past to distinguish between *L. conigenum* and *L. australe*, such as the shape of the ascomata and the presence of a pigmented basal wall (Minter 1981) are variable and should not be used to distinguish between them. This is especially true when the material originates from different hosts and areas or has been collected during different periods of the year (Ortiz-García et al. 2003; Reignoux et al. 2014).

Lophodermium indianum, a species first described from India on *P. roxburghii* Sarg., was found on samples from Colombia and is reported here for the first time on *P. maximinoi*, *P. tecunumanii* and the hybrid *P. patula* × *P. tecunumanii*. These *Pinus* species are native to countries where *L. indianum* has previously been reported, including Mexico and Guatemala (Gutiérrez-Flores et al. 2020; Salas-Lizana and Oono 2018a; Tanney and Seifert 2017). This might suggest that *L. indianum* was introduced together with material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◂**Fig. 3** *Lophodermium conigenum-australe* complex. Ascomata on the needle surface of **a** *Pinus radiata*, **b** *Pinus elliottii* × *P. caribaea,* **c** *Pinus oocarpa*, and **d** *Pinus elliottii.* Midpoint vertical section shows the **e, f** sub-hypodermal (*P. elliottii x P. caribaea*) and **g** and **h** sub-epidermal position (*P. elliottii*) of mature ascomata within the needle surface and the epidermal cells displaced at the base (arrows). **i** Asci. **j** Ascospores. **k** Black conidial masses produced in culture on FGPNA. **l** Conidia. 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 **m** MEA and **n** FGPNA. Scale bars: a–d = 500 µm; e–h = 50 µm; i = 20 µm; j = 10 µm; k = 500 µm; l = 10 µm

used to establish plantations in Colombia, as has been reported for other needle-associated fungi, including pathogens, in the past (Theron et al. 2022; Barnes et al. 2014; Janoušek et al. 2016). Roux and Lundquist (1984) reported *L. indianum* in South Africa based on morphological observations. Results of the current study for collections in South Africa call into question the reliability of that report.

The morphological data collected in this study provided no clear evidence that either of the two *L. pinastri* clades represents an existing species of *Lophodermium*. Morphologically, the available specimens from both clades produced thin, black zone lines that Minter (1981) considered characteristic of *L. pinastri* sensu stricto. In addition to this, red lips of the clypeus is a feature frequently observed for *L. pinastri* (Minter 1981). However, these were only observed for a single individual ascocarp of *L. pinastri* B, suggesting that this character is most likely not useful to delineate this species.

Phylogenetic analyses in recent studies support the view that *L. pinastri* includes cryptic taxa (Koukol et al. 2015; Salas-Lizana and Oono 2018a). Johnston et al. (2003) also identified two lineages in isolates of *L. pinastri*, one of which represented samples from Oregon and New Zealand. Results of the present study showed that this lineage also accommodates isolates from Chile and is closely related to isolates from East Asia. The isolates that we treated as *L. pinastri* B from Australia and the historical plant material obtained from the NZFRI-M in New Zealand were resolved in the second lineage that included material from Canada and countries from the Baltic Rim (Johnston et al. 2003). Recently, collections representing both these lineages have also been reported in Scotland, along with a third lineage that was confined to that region (Reignoux et al. 2014). All of these previous findings support our decision to consider isolates from Southern Hemisphere plantations as representing *L. pinastri* A and *L. pinastri* B, most likely accidentally introduced from the western United States, Canada and/or Europe (Johnston et al. 2003).

Lophodermium molitoris was originally described on *P. banksiana* Lamb. in Canada and has previously been recorded in 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), it was also found on material collected in the Bay of Plenty (Salas-Lizana and Oono 2018a). Interestingly, this species appears to have been introduced into New Zealand and not to any other country considered in this study where *P. radiata* has been established in plantations.

Previous studies have suggested that host preference and geographical distribution, together with morphological features, can be useful when identifying *Lophodermium* species (Minter 1981; Lantz et al. 2011). Furthermore, needle pathogens in the *Rhytismataceae* generally have limited host ranges and distributions where they are native as well as in areas where they have been introduced (Lantz et al. 2011; Ata et al. 2024). The results from our study provide evidence to the contrary. For example, at least three of the *Lophodermium* species occurred in two or more of the countries considered, even though their host species were different. This suggests that multiple introductions of *Lophodermium* species have occurred into Southern Hemisphere countries, where they have subsequently infected other *Pinus* species. There is also little evidence for these introduced *Lophodermium* species having spread between the studied countries.

The species of *Lophodermium* considered in this study are omnipresent endophytes of pine needles across Europe, Asia and North America (Ortiz-García et al. 2003; Salas-Lizana and Oono 2018a; Bartnik et al. 2021). An important outcome of this study is that the pathogenic species, *L. seditiosum*, was not found in collections from any of the countries sampled, even though it has been reported from Chile and South Africa in the past (Roux and Lundquist 1984; Butin and Peredo 1986;). This illustrates the fact that morphological characteristics fail to provide an acceptable approach to identify most *Lophodermium* species, at least in their introduced range, and has implications for biosecurity systems, e.g. risk assessments.

The presence of *Lophodermium* species in several Southern Hemisphere countries was confirmed for the first time using DNA sequence data that enriches the available database for *Lophodermium* species. Additionally, this study showed that the *TEF*1 gene region amplified relatively well for different *Lophodermium* species and that it is a promising marker for future phylogenetic studies. A caveat is that several of the taxa have yet to be clearly delimited. This is particularly challenging because these fungi do not produce sexual fruiting bodies in culture and there is a significant

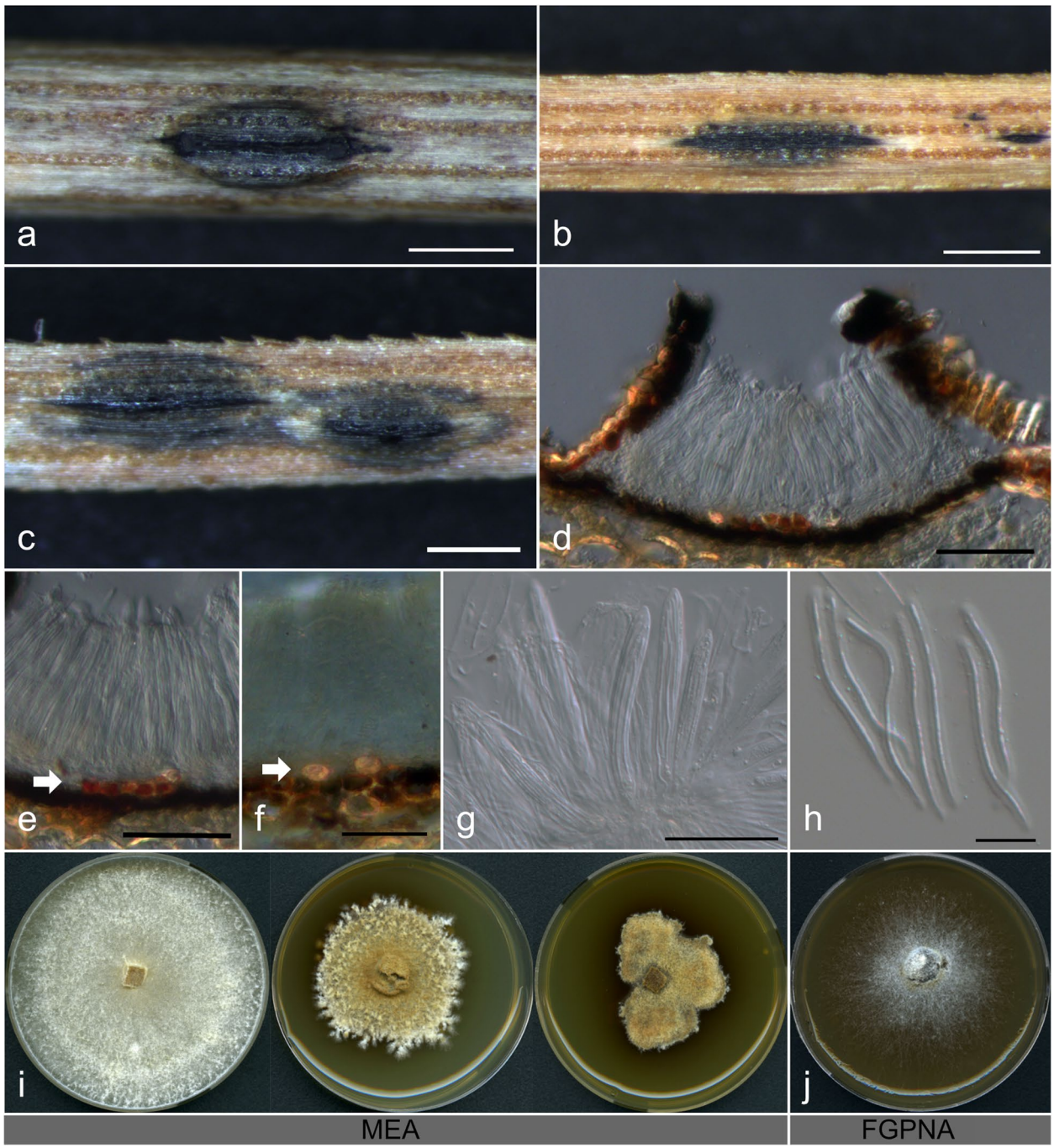

Fig. 4 *Lophodermium indianum* collected from Colombia. Ascomata on the needle surface of **a** *Pinus patula*, **b** *Pinus tecunumanii*, and **c** *Pinus maximinoi*. Midpoint vertical section shows the sub-hypodermal position of the ascomata in the needle surface and the cells displaced at the base in **d, e** *Pinus patula* and **f** *Pinus maximinoi* (arrow). **g** Asci. **h** Ascospores. Variability observed in the culture morphology of eight-week-old colonies for isolates CMW55987, CMW56850, and CMW56987 grown on **i** MEA and **j** FGPNA. Scale bars: a–c = 500 µm; d–h = 50 µm

lack of cultures specifically linked to needle specimens bearing these structures. A robust future taxonomy of *Lophodermium* species will require focused collections of these fungi, and other *Rhytismatales*, on conifers in the many areas where their host trees are native.

All *Pinus* species in the Southern Hemisphere are non-native, where they have mainly been introduced for plantation forestry. Unlike, for example, introduced *Eucalyptus* species where numerous pathogen host-shifts have occurred from native trees belonging to the related

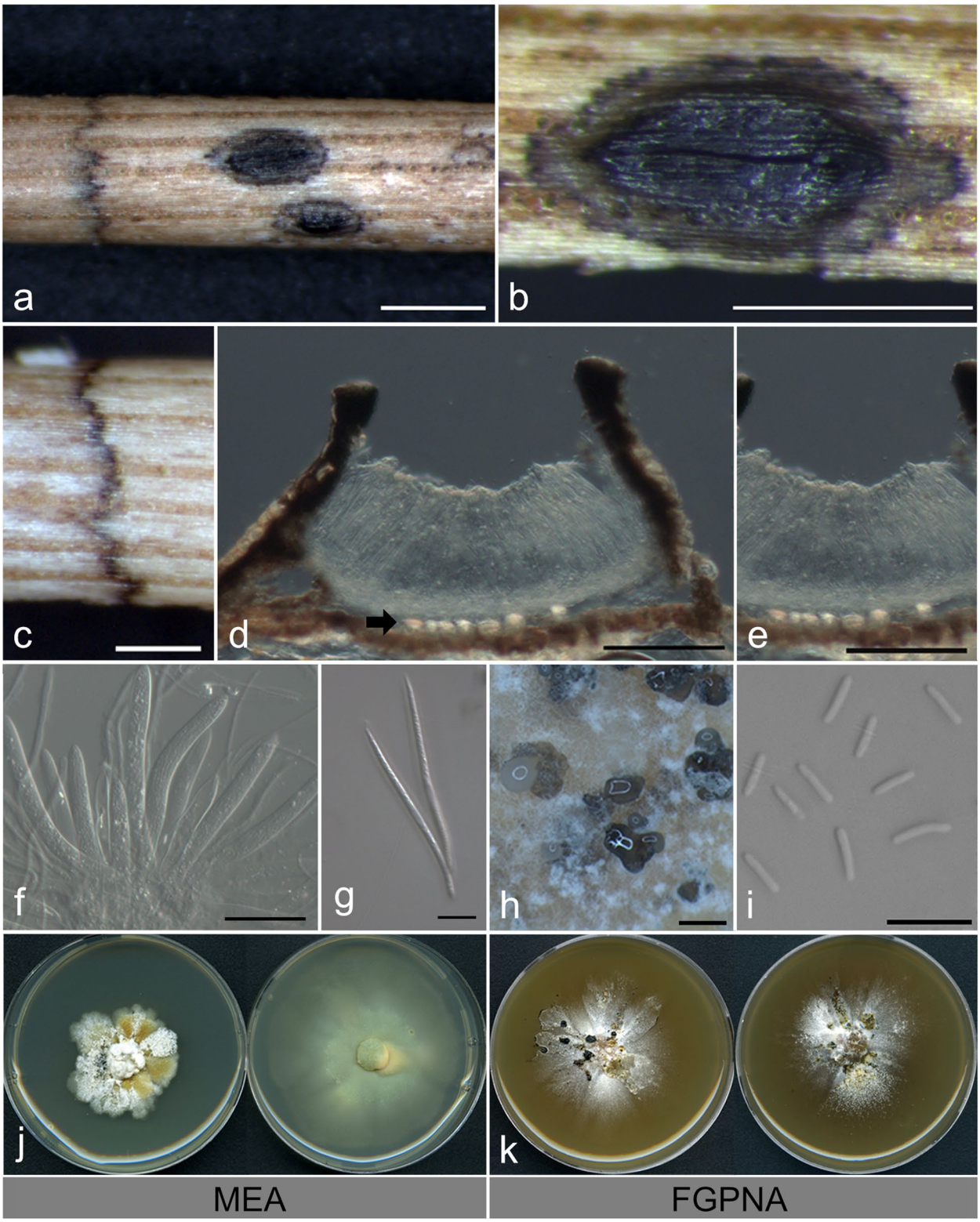

Fig. 5 *Lophodermium pinastri* A collected in Chile and New Zealand. **a, b** Ascomata and **c** black zone lines observed on the needle surface of *P. radiata*. **d, e** Midpoint vertical section shows the subepidermal ascomatal position in the needle surface of *P. radiata,* and the displaced epidermal cells grouped at the base (arrow). **f** Asci. **g** Ascospores. **h** Conidial masses. **i** Conidia. Variation observed in the colony morphology of eight-week-old colonies grown on **j** MEA and **k** FGPNA for isolates from Chile representing subclade 2 (CMW58475) and New Zealand representing subclade 1 (CMW57330), respectively. Scale bars: a–b = 500 µm; c = 200 µm; d = 50 µm; e = 20 µm; f = 50 µm; g = 10 µm; h = 500 µm; i = 10 µm

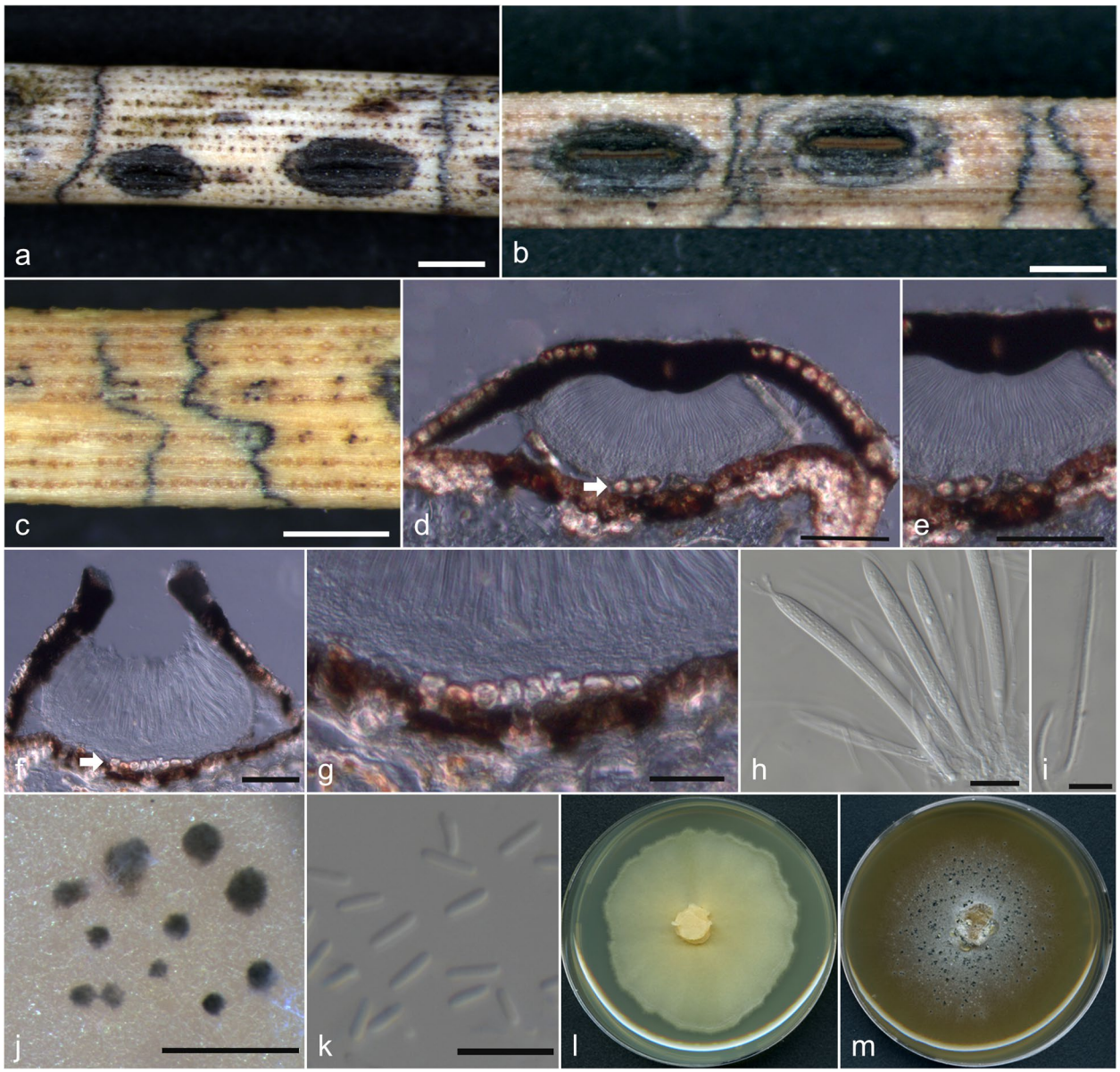

Fig. 6 *Lophodermium pinastri* B collected in Australia. Ascomata on the needle surface of **a** *Pinus ponderosa* and **b** *Pinus patula,* with black and red lips, respectively. **c** Black zone lines observed on all samples analysed. Midpoint vertical section shows the positioning of the ascomata 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. **j** Black conidial masses observed on the surface of FGPNA. **k** Conidia. Eight-week-old colony grown on **l** MEA and **m** FGPNA for an isolate from Australia (CMW56855). Scale bars: a–c = 500 µm; d–g = 50 µm; h = 20 µm; i = 10 µm; j = 500 µm; k = 10 µm

Myrtales (Slippers et al. 2005; Burgess and Wingfield 2016; Suzuki et al. 2022), introduced *Pinus* species have no close relatives in the Southern Hemisphere. Consequently, the primary host-specific pathogens of *Pinus* species, including the *Lophodermium* species considered in this study, have all apparently been introduced from the native ranges of these trees (Gibson 1979; Raffa et al. 2023). The pathogen 'spill-over' effect seen with trees in the *Myrtales* and *Fabales* (Crous et al. 2017; Raffa et al. 2023) is not found for *Pinus* species. Unexpectedly, our study revealed that some *Lophodermium* species exhibit low host specificity in introduced regions, infecting *Pinus* species different from those of their native hosts. This could imply adaptation of these fungi with unknown consequences as they potentially move to new environments or even back to their areas of origin.

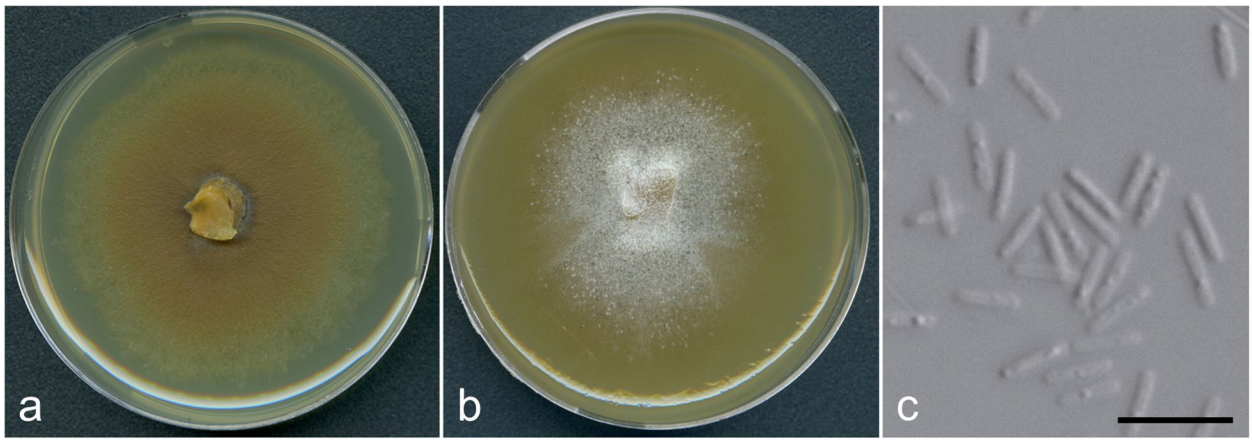

Fig. 7 *Lophodermium molitoris* collected in New Zealand. Eight-week-old colony of isolate CMW57341 grown on **a** MEA and **b** FGPNA. **c** Conidia. Scale bar: c = 10 µm

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Author contribution Conceptualization: I. Barnes and M.J. Wingfield; methodology: C.A. Theron; performing the experiments: C.A. Theron; resources: I. Barnes, M.J. Wingfield, R. Ahumada, A.J. Carnegie, S. Fraser, C.A. Rodas; writing—original draft preparation: C.A. Theron; writing—review and editing: I. Barnes, M.J. Wingfield, R. Ahumada, A.J. Carnegie, S. Fraser, C.A. Rodas; supervision: I. Barnes, M.J. Wingfield; project administration: I. Barnes; funding acquisition: I. Barnes, M.J. Wingfield, R. Ahumada, A.J. Carnegie, S. Fraser, and C.A. Rodas. All authors have read and agreed to the published version of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applicable.

Consent to participate All authors participated in this work.

Consent or publication All authors agreed to publish this work.

Competing interests The authors declare no competing interests.

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