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ORIGINAL ARTICLE

Blast from the past: a study of decades-old fungal cultures resolves a long-standing tree disease mystery

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Abstract
A root disease in plantations of *Pinus radiata* and *Pinus pinaster*, where trees died in distinct patches, was present in the Western Cape province of South Africa during the 1970s and 1980s. *Phytophthora cinnamomi* was initially believed to be the cause, but the disease was later ascribed to the insect-associated fungus *Leptographium serpens*, a fungal species residing in the Ophiostomatales. Doubt regarding the cause of the disease was raised in a later study due to the fact that most *Leptographium* spp., particularly those that colonise ray parenchyma tissues, which is the case for *L. serpens*, are not typically primary disease agents. In this study, cultures of an unidentified sterile fungus collected from the dying trees were revived and identified using DNA sequencing methods, which were not available when the disease was first studied. These cultures were identified as the pyrophillic pathogen *Rhizina undulata*, well-known to cause patch death of conifers in South Africa and elsewhere in the world. While the patches of dying trees no longer exist and the disease cannot be newly studied, it is most likely that the tree death originally thought to be caused by *L. serpens* was due primarily to *R. undulata*. The study provides a vivid example of the value of preserving cultures of fungi for later study and the power of modern techniques to identify fungal pathogens.

Keywords *Rhizina undulata* · *Leptographium* · Ophiostomatales · Tree disease · Disease diagnostics

Introduction

During the 1970s and 1980s, patches of *Pinus radiata* and *Pinus pinaster* growing in the Grabouw area of South Africa's Western Cape province were observed to be diseased (Fig. 1). The disease was typified by trees at the peripheries of these patches gradually dying, while naturally regenerating plants within the dead areas wilted and died during the first few years of growth. At the time when this disease was first observed, the Oomycete root pathogen *Phytophthora cinnamomi* was recognized as important to agriculture and particularly the Protea (Proteaceae) cut-flower industry of the Western Cape province (Brits et al. 1983). An interest in *P. cinnamomi* also led to its discovery in a Grabouw forestry nursery (Donald and Broembsen 1977). And in turn, the patches of diseased trees in local plantations were assumed to be due to that pathogen (Wilson 1977).

The dying trees found in the disease centres showed evidence of root disease with actively developing lesions in the cambium of the roots and root collars. Infected wood associated with cambial infection had a distinct grey to black colour that did not penetrate into the wood. No fungal fruiting bodies were found on the ground associated with the dying trees. A fungus was consistently isolated from the leading edges of the actively developing lesions on the diseased roots. It was determined that this fungus was a new species in the genus *Verticicladiella* and the name *Verticicladiella alacris* was provided for it (Wingfield and Marasas 1980). Pathogenicity tests with the fungus showed that it gave rise to lesions similar to those found on dying trees, and the disease was subsequently attributed to this fungus (Wingfield and Knox-Davies 1980).

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Fig. 1 (A-C) Dying *Pinus pinaster* trees in the Grabouw plantation, Western Cape Province, South Africa. (B) Infected trees in distinct patches with newly dying trees at the periphery. (C) Naturally regenerated trees within a disease centre dying, as indicated by white arrows. Note: these photographs were taken between 1976 and 1979 in a plantation area that no longer exists. (D) Ascocarp of *Rhizina undulata* commonly found in South African *Pinus* plantations after fire. These were not found during the disease outbreak in the 1970s and 1980s. (E) Asci and ascospores of *R. undulata* from a South African collection of the pathogen. Scale bar: E = 100 µm

The description of *V. alacris* relied on morphological characteristics (Wingfield and Marasas 1980). This also led to the fungus being reduced to synonymy with *Verticicladiella serpens* (Wingfield and Marasas 1981), a fungus associated with diseased roots of *Pinus pinea* in Italy (Lorenzini and Gambogi 1976). Later, these fungi and other species of *Verticicladiella* were transferred to the genus *Leptographium*, a well-represented asexual genus of the Ophiostomatales (Seifert et al. 2013; De Beer et al. 2022). The fungus originally associated with dying *Pinus* spp. in South Africa was relegated to the name *Leptographium serpens* (Wingfield 1985). Duong et al. (2012) conducted a detailed study of *Leptographium* spp. associated with root infections of *Pinus* spp. in which DNA sequence data for five gene regions were applied. That study showed that the fungus associated with the root disease of the *Pinus* spp. in the Western Cape was different to *L. serpens* and the name of the South African fungus was most appropriately *Leptographium alacre* (Morelet 1985; Duong et al. 2012; De Beer et al. 2022).

There has been a long-standing debate regarding the role that *Leptographium* spp. can play as the primary causal agents of conifer root disease (Harrington 1988; Wingfield et al. 1988; Jacobs and Wingfield 2001). Three *Leptographium* spp. have been associated with such diseases including the three varieties of *Leptographium wageneri* (Wagener and Mielke 1961), *Leptographium procerum* (Dochinger 1967) and, as mentioned above, *L. serpens* (Lorenzini and Gambogi 1976). Of these fungi, *L. wageneri*, the causal agent of black stain root disease is clearly different to the others. This difference lies in the fact that it colonises only the tracheids and does not invade the ray parenchyma tissues (Diamandis et al. 1997; Harrington and Cobb Jr 1983; Wingfield 1986). In this regard, it might be considered a true vascular wilt pathogen. It is thus very different to other species of *Leptographium* that colonise the ray parenchyma tissues, giving rise to pie-shaped lesions similar to the so-called blue-stain fungi (Jacobs and Wingfield 2001). Such pie-shaped discoloration was not seen in diseased trees from the 1970s and 1980s outbreak, because

the activiely infected tissue was in the fresh cambium and would not have developed at this early state of infection. The role of these other *Leptographium* spp. in root disease is best considered as contributing to tree death rather than being primary disease agents (Jacobs and Wingfield 2001). This then calls to question the role that *L. serpens* played in causing the root disease of *Pinus* spp. in the Western Cape province of South Africa in the 1970s and 1980s.

Wingfield et al. (1988) raised the issue that the root disease of *P. radiata* and *P. pinaster* in South Africa had not been fully elucidated. In that review, it was mentioned that a sterile fungus, tentatively referred to as a species of *Rhizoctonia*, had been isolated from the roots of the trees in question, including those from which *L. alacre* had been isolated. The present study considered the identification of cultures of the sterile fungus isolated in 1984 and referred to by Wingfield et al. (1988), which have been preserved in a culture collection for approximately four decades.

Materials and methods

Cultures

This study relied on the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) housed at the University of Pretoria, Pretoria, South Africa. Four cultures were deposited in the collection in March 1987 by the first author (MJW) of the present paper and were isolated from the roots of dying trees at the periphery of the disease centres and from the roots of the young naturally regenerating trees within the patches. Two of these cultures (CMW 435 and CMW 436) were isolated from *Pinus pinaster* on September 11, 1984 and two (CMW 413 and CMW 412) were isolated from *P. radiata* on July 17 and August 8, 1984, respectively. These four cultures were accessed from the collection in August 2020 and plated on 2% malt extract media (20 g Biolab malt extract, 20 g Difco agar, 1 L deionized water). They were incubated in the dark at room temperature and two cultures (CMW 412 and CMW 436) could be revived.

PCR, DNA sequencing, and phylogenetic analyses

DNA was isolated from 7-day-old cultures of isolates CMW 412 and CMW 436 using the Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific). The internal transcribed spacer regions 1 and 2 (ITS), including the 5.8 S rRNA region, were amplified using primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). PCR mixtures were prepared following the protocols described by Pham et al. (2019). The thermal cycling included an initial denaturation at 95 °C for 5 min followed by 10 primary amplification cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C, then 30 additional cycles of the same reaction sequence, with a 5 s increase in the annealing step per cycle, and the reactions were completed with a final extension at 72 °C for 10 min. Amplified fragments were treated with ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific). The purified products were sequenced in both directions using the BigDye terminator sequencing kit v. 3.1 (Thermo Fisher Scientific) on an ABI Prism 3100 DNA sequencer (Thermo Fisher Scientific) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. Geneious Prime v. 2022.1.1 (https://www.geneious.com) was used to assemble and trim the raw sequences, which were deposited in GenBank (Table 1).

Preliminary identification was made by performing a nucleotide BLAST search using the ITS sequences against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) to identify the isolates to the genus level. Sequences of species closely related to those emerging from this study were sourced from the GenBank database (Table 1). All sequences were aligned with MAFFT v. 7 (Katoh and Standley 2013) and inspected manually using MEGA v. 7 (Kumar et al. 2016). A Maximum-Likelihood (ML) analysis was conducted using RaxML v. 8.2.4 (Stamatakis 2014) on the CIPRES Science Gateway 3.3 (Miller et al. 2011), with default GTR substitution model and 1000 rapid bootstraps. Sequences for *Helvella bachu* (HKAS 88105) and *Helvella calycina* (O-253255) were used as outgroups.

Results

PCR, DNA sequencing, and phylogenetic analyses

For both of the South African isolates considered in this study, amplicons of approximately 500 bp were generated for the ITS region. The dataset used in the phylogenetic analyses included 15 ingroup taxa and contained 801 characters, including alignment gaps. When comparing sequences for the isolates considered in this study with those for species in Rhizinaceae, the two South African isolates had identical sequences and formed a well-supported (95%) monophyletic clade in the ML tree (Fig. 2) with all the reference isolates of *Rhizina undulata*. The isolates were thus identified as that fungus.

Table 1 Collection details and GenBank accessions of the isolates included in the phylogenetic analyses

Species	Isolate	Host/substrate	Country	ITS accession number	References
Rhizina undulata	BPL929	Burned ground in *Pinus* sp. forest	USA	MF992154	Healy et al. (2021)
Rhizina undulata	KW_39968	N/A*	Ukraine	KC201234	Healy et al. (2021)
Rhizina undulata	JLF6721	N/A	USA	MK863520	Healy et al. (2021)
Rhizina undulata	PDS-5	*Pinus densiflora*	South Korea	EU346951	Lee et al. (2007)
Rhizina undulata	WB1059	Endolichenic (terricolous/ musicolous lichen)	Canada	MZ091970	Healy et al. (2021)
Rhizina undulata	QU0170	Endophytic (lycophyte)	Canada	MZ091969	Healy et al. (2021)
Rhizina undulata	PDK-1	*Pinus densiflora*	South Korea	EU339123	Lee et al. (2007)
Rhizina undulata	TENN-F-069463	Burnt wood on ground	USA	MN047434	Hughes et al. (2020)
Rhizina undulata	CBS 300.56	soil under *Picea pungens*	Netherlands	MH857650	Vu et al. (2019)
Rhizina undulata	**CMW 412****	*Pinus radiata roots*	South Africa	OR580960	*This study*
Rhizina undulata	**CMW 436****	*Pinus pinaster roots*	South Africa	OR580961	*This study*
Psilopezia deligata	RH1660	N/A	USA	MT374020	Healy et al. (2021)
Psilopezia deligata	KH.99.13	N/A	USA	EF494044	Healy et al. (2021)
Phymatotrichopsis omnivora	POD13	*Gossypium hirsutum*	USA	KJ410123	Chitrampalam and Olsen (2014)
Phymatotrichopsis omnivora	POD11	*Gossypium hirsutum*	USA	KJ410121	Chitrampalam and Olsen (2014)
Helvella calycina	O-253255	N/A	Norway	MN656158	Skrede et al. (2017)
Helvella bachu	HKAS:88105	Soil under *Populus euphratica*	China	KU739791	Zhao et al. (2016)

* N/A: Not available

** The isolates indicated in boldface were those withdrawn from the Forestry & Agricultural Biotechnology Institute's culture collection (CMW).

Discussion

An unknown sterile fungus, isolated from the roots of diseased *Pinus* spp. in South Africa and thought to be dying due to infection by the Ophiostomatoid fungus *L. alacre*, was identified in the present study as the root pathogen *R. undulata*. This fungus is well-known to cause patch death of *Pinus* spp. as well as other conifers in various parts of the world (Peace 1962; Gibson 1979). It is also well-known in South Africa (Fig. 1), where it can result in a failure to establish new plantations after fire (Gibson 1979; Lundquist 1984). The previous uncertainty (Wingfield et al., 1988) regarding the role of *L. alacre* in causing the patch death of *P. radiata* and *P. pinaster* is thus likely resolved by the results of this study. This arises from the fact that we have identified a well-known root pathogen, typically sterile in culture, that was isolated from actively emerging lesions on the roots of the dying trees, some 40 years after the disease problem was first reported.

An important question arising from this study is what role *L. alacre* might have played in the root disease as reported by Wingfield and Marasas (1980). It is not possible to consider this question experimentally due to the fact that the plantations where the disease occurred no longer exist. However, a plausible explanation lies in the biology of *L. alacre* and the manner in which it spreads. This fungus is well-known to be vectored by the root-feeding scolytine (Coleoptera: Curculionidae: Scolytinae) beetle *Hylastes angustatus* in South Africa (Wingfield and Knox-Davies 1980; Wingfield and Marasas 1980; Zhou et al. 2001). These beetles undergo maturation feeding on the roots of living *Pinus* trees before they colonise stressed or dying trees. During this feeding period, they transmit their fungal associates, including *L. alacre*, to the roots. The inoculation trials, conducted in the study of Wingfield and Knox-Davies 1980, showed that *L. alacre* was able to induce lesions on *P. pinaster* and *P. radiata* and that it had relatively high levels of aggressiveness. It is thus likely that *L. alacre* would have been introduced into these roots due to beetle maturation feeding, outcompeting other fungi including *R. undulata*, which we now believe was the primary pathogen.

R. undulata is a pyrophillic fungus, requiring heat shock for ascospore germination and for the fungus to become an active pathogen (Jalaluddin 1967). It was first discovered in Europe in the late 1800s and was thought to be the causal agent of *la maladie due ronde* in France and *Ringseuche* in Germany (Gremmen 1971). It has commonly been referred to as the "coffee fire fungus" because conifer trees would die in patches activated by the heat of camp fires made to prepare tea or coffee on forestry trails (Peace 1962; Gremmen

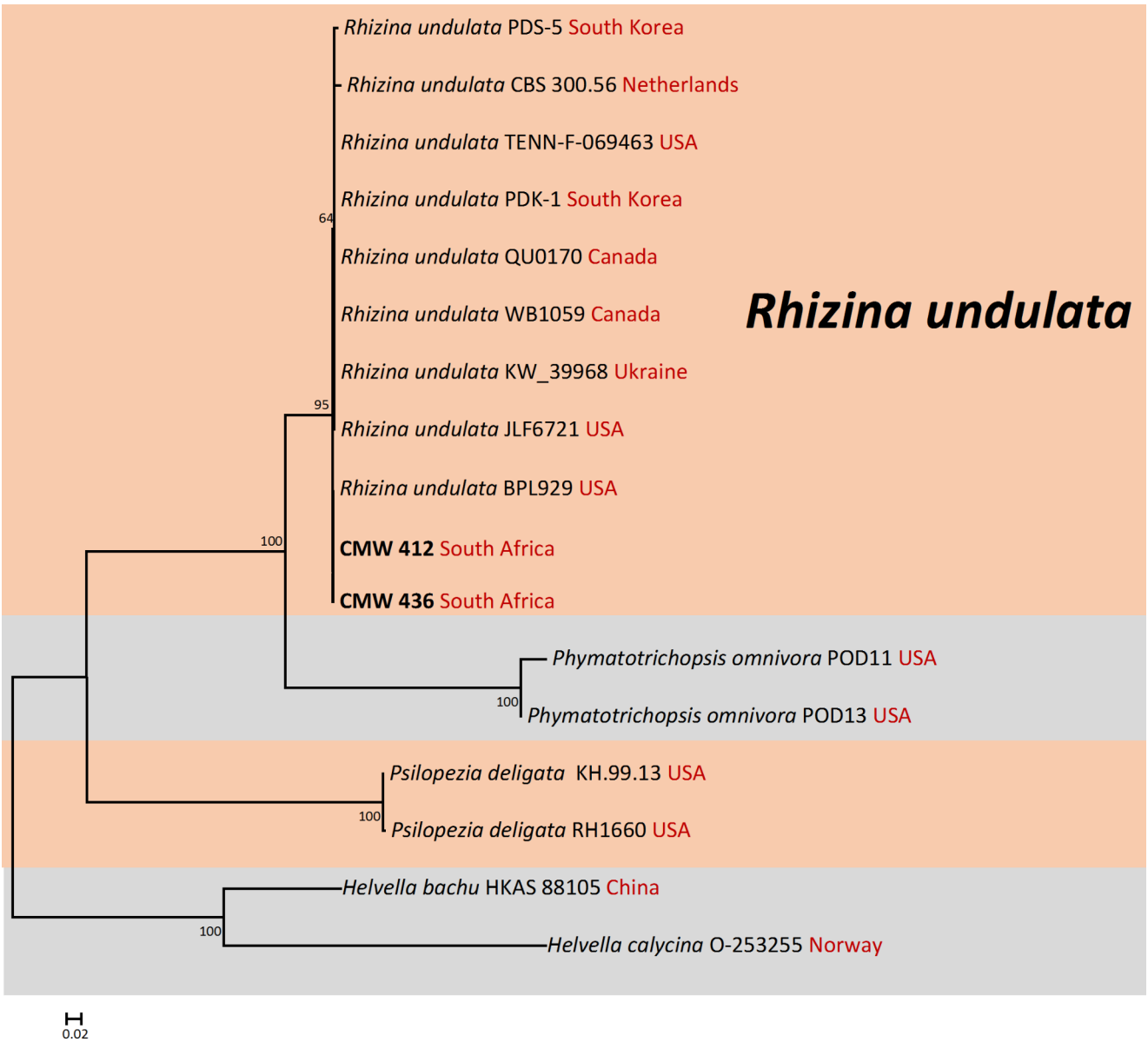

Fig. 2 Phylogenetic tree based on a Maximum Likelihood (ML) analysis of ITS sequences representing species of Rhizinaceae. The two isolates collected in South Africa in 1984 and deposited in the culture collection (CMW) of FABI are presented in **bold** face. The countries of origin of the isolates are indicated in red text. Bootstrap values for the ML analyses are indicated at the nodes. *Helvella bachu* (HKAS 88105) and *Helvella calycina* (O-253255) represent the outgroup taxa

1971; Gibson 1979). Thus, an important question arising from the present study is what form of heat stimulus would have activated the pathogen and initiated *P. radiata* and *P. pinaster* patch death, the disease previously attributed to *L. serpens*.

Forms of heat-shock other than forest fires, such as hot coals being thrown from steam locomotives, hot asphalt being laid for the construction of new roads, and accidental fires have been recorded to result in active *R. undulata* disease centers (Gremmen 1971). Damage due to *R. undulata* in South Africa is mostly associated with burning of forest waste after clear-felling or accidental fires in plantations of *Pinus* spp. (Lundquist 1984; Wingfield and Swart 1994). There was no evidence of fires having occurred in any of the patches of dying trees in the Western Cape pine plantations, which would have otherwise provided an early explanation for the problem. While *R. undulata* typically requires a heat shock to be activated, there is limited evidence to suggest that it can become active in highly acidic soils and in the presence of living conifer roots (Jalaludin 1967). Soils in the areas where the disease was present, known as Table Mountain sandstone (Richards et al. 1997), are sandy and

acidic. This provides at least anecdotal support for the fact that *R. undulata*, which is well-known in Western Cape pine plantations, could have infected the roots of the trees in the absence of a heat shock.

An interesting and important manifestation of the root disease attributed to *L. serpens* (now *L. alacre*) was the fact that naturally regenerated trees within the disease patches became diseased and wilted up to approximately one year of age (Fig. 1; Wingfield and Knox-Davies 1980; Wingfield et al. 1988). These trees had active lesions in the freshly infected tissues and from which *L. serpens* was easily isolated. A plausible explanation for the continuous death of the naturally regenerating trees could relate to the biology of *H. angustatus* (Tribe 1990). Populations of these insects would have built up in the older dying trees at the periphery of the patches. These insects would then be attracted to the roots of the young naturally regenerating trees where they would undergo maturation feeding and together with *L. alacre*, cause the young trees to die. Whether these young trees within the disease centres had also died due to *R. undulata* infection remains unclear.

The fact that the cause of tree death in patches was first attributed to *P. cinnamomi* was incorrectly based on an intense interest in that pathogen at the time. This view would furthermore have been supported by the fact that *P. cinnamomi* had been found in a nursery providing planting stock for the plantation where the disease occurred (Brits et al. 1983; Donald and Broembsen 1977) and reports of *P. radiata* dying in other parts of the world (Newhook 1959; Gibson 1979). Attributing the disease to a species of *Leptographium* was also erroneous and arose from the consistent association of *L. serpens* with early disease symptoms and the results of pathogenicity tests with the fungus. If the non-sporulating (sterile) cultures isolated from the roots of dying *P. pinaster* and *P. radiata* could have been identified at the time, the cause of the disease would likely have been described differently. Importantly, the results of the present study relied on DNA-based sequencing technology that was not available at the time that the disease problem was first observed. This illustrates the importance of such advancing technologies in the fields of forest pathology and tree health.

This study would not have been possible without access to the unidentified cultures that had been preserved for approximately four decades. Furthermore, the results have provided an explanation for an unexplained tree disease problem; one that has confused the literature regarding *Leptographium* root diseases for many years. Importantly, it highlights the significance of fungal culture collections and our need to preserve cultures for long periods of time, regardless of their perceived importance at their time of collection. There are few formally recognized and financially subsidized culture collections globally and none of these would consider preserving cultures of unnamed fungi such as those that made the present study possible. The question thus arises as to whether novel approaches, including new technologies, should not be considered such that larger and more diverse living fungal cultures could be maintained for future study. Amongst other advantages, this would advance the study of microbes including fungi, beyond a largely comparative taxonomic focus.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-023-01502-1.

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Author contributions MJW, BDW and AW conceived and designed the study ; NQP and TAD executed the sequencing and phylogenetic analyses and SM maintained cultures. MJW wrote the first draft of the paper and all authors contributed to its completion.

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Data availability The sequences that were generated for this study have been deposited at the NCBI. The accession numbers will be made available upon publication.

Declarations

Statements & declarations The authors have no competing interests to declare that are relevant to the content of this article.

Conflict of interest The authors have no conflicting interests to declare that are relevant to the content of this article.

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