African Entomology 2024, 32: e18505 (8 pages)
https://doi.org/10.17159/2254-8854/2024/a18505
RESEARCH ARTICLE

AFRICAN ENTOMOLOGY
ISSN (online) 2224-8854

Bark beetles and their associated fungi infesting native *Widdringtonia* species in the Western Cape province of South Africa

Renier Johannes Basson[1], Francois Roets[1], Michael J. Wingfield[2], Janneke Aylward[1,2*]

[1] *Department of Conservation Ecology and Entomology, Stellenbosch University, Matieland, South Africa.*
[2] *Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Hatfield, South Africa.*

Widdringtonia is a genus of native southern African Cupressaceae trees comprising two species that occur in the mountains of the Western Cape province, South Africa. *Widdringtonia cedarbergensis* has a localised distribution and is critically endangered, while *W. nodiflora* is widespread and common. Little is known regarding the biotic associations of these trees. The aim of this study was, consequently, to identify bark beetles (Curculionidae: Scolytinae) and their associated fungi on *Widdringtonia* species in the Western Cape. Bark beetles were collected from infested *W. cedarbergensis* at three different locations in the Cederberg and from *W. nodiflora* at one site on the Franschhoek Pass. Beetle identification was based on morphology and sequence data of the *COI* gene region. Fungi were isolated from beetles, their frass and the walls of their tunnels and grouped according to morphology. Morphogroups were identified by sequencing the ITS region of representative isolates. Four phylogenetically closely related bark beetle species residing in the genus *Lanurgus* (Micracidini) were identified, three from *W. cedarbergensis* stem sections, twigs and cones, respectively, and one from *W. nodiflora* stems. Of these, only the *W. cedarbergensis* twig beetle is of a previously described species and is currently known as *Diplotrichus widdringtoniae*. *Piskurozyma* sp. (Tremellomycetes) and *Yamadazyma* sp. (Saccharomycetes) yeasts were most closely associated with *D. widdringtoniae* (*Lanurgus* sp. 1) and *Lanurgus* sp. 2 beetles, whereas *Geosmithia* spp. (Sordariomycetes) had a strong association with *Lanurgus* sp. 3 and *Lanurgus* sp. 4. This is the first comprehensive report of bark beetles and their associated fungi infesting *Widdringtonia*.

CORRESPONDENCE
Janneke Aylward

EMAIL
janneke.aylward@fabi.up.ac.za

DATES
Received: 24 April 2024
Accepted: 1 August 2024

KEYWORDS
Cupressaceae
cedar
Geosmithia
Lanurgus
symbiosis
yeast

SUPPLEMENTARY MATERIAL
Supplementary information for this article is available separate to this pdf

INTRODUCTION

Widdringtonia is the only genus of Cupressaceae that is native to southern Africa where it is confined to high-altitude mountainous areas (Pauw, Linder 1997). In South Africa, the Mountain cedar *W. nodiflora* is the most common and widespread species (Farjon, Filer 2013). In contrast, the Clanwilliam cedar, *W. cedarbergensis* (syn. *W. wallichii*), and Baviaanskloof cedar, *W. schwarzii*, are highly localised and endemic. *Widdringtonia cedarbergensis* is critically endangered due to both anthropogenic and ecological threats (Farjon et al. 2013; White et al. 2016) where one of the greatest contributors to its decline is historical over-harvesting (Hubbard 1937; Smith 1955). This species is also ecologically and biologically anomalous in the fynbos landscape because it is poorly adapted to fire, lacks a canopy-stored seed bank, and grows slowly, taking many years to reach maturity (Manders 1986).

Insect associates of *Widdringtonia* have not been well documented. Those associated with the wood include bark beetles (Coleoptera: Scolytinae) and a jewel beetle, *Acmaeodera glabella* (Coleoptera: Buprestidae; Wingfield et al. 1988). Two bark beetles, *Diplotrichus widdringtoniae* (syn. *Lanurgus widdringtoniae*) and *Afrocleptus widdringtoniae*, have been reported from dead and dying *W. cedarbergensis* (Wingfield et al. 1988). A third bark beetle, identified as *Eidophelus usagaricus* (syn. *Cryphalomorphus usagaricus*), infests *W. cedarbergensis* cones (Botha 1990). This species feeds on the scales of recently opened mature female cones and expedites the release of *W. cedarbergensis* seeds between fire regimes (Botha 1988). A secondary inhabitant, the weevil *Himatium variolosum* (Curculionidae: Cossoninae), has been found in old cones (Botha 1990). Despite these reports, no targeted survey of insect species associated with *Widdringtonia* has been undertaken and recent advances in the taxonomy of the Scolytinae (Hulcr et al. 2015a) and a lack of reference material may make these previously reported associations ambiguous due to potential misidentification.

Similar to the insects, microbial associates of *Widdringtonia* have received little attention. A preliminary study (Wingfield et al. 1988) found several microbes associated with the roots, stems, and leaves of unhealthy *W. cedarbergensis*. These included root rot of unknown cause and the invasive root-pathogen, *Phytophthora cinnamomi*. Fungi isolated from leaves in that study included a *Meliola* sp. from healthy leaves and a *Phomopsis* sp. from dead leaves and twigs. Cankers and brown cubical rot were associated with fire scars on trees. Microbial associates of *W. nodiflora* are similarly poorly studied but cypress canker, caused by the fungus *Seiridium neocupressi*, was recently discovered in a natural population of these trees (Wingfield et al. 2022b). This lack of knowledge highlights an urgent need for attention to be paid to pests and pathogens of native *Widdringtonia* species.

Most bark beetle species form symbiotic associations with fungi (Hulcr et al. 2020). These associations are of particular importance because they include some of the most important causes of tree death worldwide (Ploetz et al. 2013). For example, various elm bark beetles in the genus *Scolytus*, but particularly *S. scolytus* and *S. multistriatus*, spread the Dutch Elm Disease fungi *Ophiostoma ulmi* s.l. (Santini, Faccoli 2015). This association has devastated native *Ulmus* spp. tree populations in Asia, Europe and

North America. There are also bark beetle-fungal symbioses for which the nature of the interaction has not yet been determined. For example, species of *Geosmithia* are often dominant in the galleries of subcortical beetles. While few have been described as obligate ambrosial symbionts (Kolařík, Kirkendall 2010), most are consistently associated with either a limited range or a wide variety of bark beetles (Meshram et al. 2022; Pepori et al. 2015). However, the relevance of these associations is generally unknown (Kolařík, Hulcr 2023). Other beetle-fungal interactions may be merely co-incidental.

In South Africa, most studies on beetle-fungus interactions have focused on fungal associates of non-native bark beetle species accidentally introduced and infesting plantation-grown *Pinus* species (Tribe 1991; Wingfield et al. 2001; Wingfield, Swart 1994). In contrast, bark beetles infesting native trees have received only limited attention (Machingambi et al. 2014; Musvuugwa et al. 2015; Van der Linde et al. 2016). Other than the two studies reporting bark beetles on *W. cedarbergensis* 30 years ago (Botha 1990; Wingfield et al. 1988), no data are available regarding bark beetles infesting *Widdringtonia* trees. That the microbial associates in these systems have not been investigated is thus not surprising. The critically endangered nature of *W. cedarbergensis* implies that the organisms associated with the trees may also be endangered. The aim of this study was, therefore, to document the Scolytinae and their associated fungi from *W. cedarbergensis* and *W. nodiflora* in the Western Cape Province of South Africa.

MATERIALS AND METHODS

Bark beetle sampling and identification

Bark beetles were sampled from trees at multiple locations in the Western Cape Province of South Africa (Figure 1). Infested material of *W. cedarbergensis* was sampled in Algeria (32°21′53″ S, 19°04′10″ E), Welbedacht (32°24′40″ S, 19°10′28″ E) and De Rif (32°26′21″ S, 19°11′17″ E) in the Cederberg mountains. Sampling of *W. nodiflora*-associated bark beetles took place at one site on the Franschhoek Pass (33°56′11″ S, 19°09′44″ E).

Individual trees were visually inspected for signs of infestation, such as active beetle galleries. Infested areas were removed and placed in emergence cages from which beetles could be collected. For a subset of the material, beetle individuals were collected directly from galleries by scraping or cutting away the bark and wood. The attraction of Scolytinae to stressed or dying host trees was also exploited by placing five to 10 freshly cut stem sections around beetle-infested trees in spring, when Scolytinae become active. These stem sections (*ca.* 10 cm thick and 50 cm long) were cut from healthy *W. nodiflora* and *W. cedarbergensis* trees, their ends sealed with paraffin wax and placed under *W. cedarbergensis* trees in the Cederberg. At the same time, a branch on *ca*. five trees per population was cut and left under the trees. After four weeks, these cut stems and branches were placed in emergence cages, with a subset used for direct collection as described above.

A subset of all beetles collected directly from the wood samples and from emergence traps were stored in 98% ethanol at –80°C for identification and reference purposes. Beetles were inspected and photographed using a Leica MZ16 A microscope and camera. Morphological identification to tribe and genus level were made using the key of Jordal (2021c). Classification to species level was made with assistance from taxonomic experts (Dr A Johnson, University of Florida, and Dr BH Jordal, University of Bergen) and by sequencing the cytochrome c oxidase subunit 1 mitochondrial (*COI*) gene region. Dissected heads of selected individuals for each tentatively identified species were used for DNA extraction with the Macherey Nagel NucleoSpin Tissue Kit (Macherey Nagel, Düren, Germany). Amplification using the primers LCO-1490 and HCO-2198 (Folmer et al. 1994) followed previously described protocols (Hebert et al. 2003) and products were sequenced at the Central Analytical Facilities (CAF), Stellenbosch University. The sequences have been deposited in GenBank under the accession numbers PP744484–PP744487.

For phylogenetic analysis, the *COI* sequences of related species were obtained from a recent taxonomic assessment of the Micracidini (Jordal 2021c). Sequences were aligned with MAFFT v 7.525 (Katoh, Standley 2013) and trimmed with trimAl v1.4 (Capella-Gutiérrez et al. 2009). After choosing an appropriate DNA substitution model with ModelTest-NG v 0.1.7 (Darriba et al. 2020), a Maximum Likelihood (ML) tree was determined with RAxML-NG v 1.2.1 (Kozlov et al. 2019) using the TVM+I+G4 model. The tree was exported to iTOL v 6 (Letunic, Bork 2021) for viewing and editing.

Fungal isolation and identification

Three sets of Malt Extract Agar (MEA; Biolab, South Africa) selective media, supplemented with different combinations of antimicrobials (Sigma-Aldrich, St Louis, USA) were used to isolate fungi from the collected beetles. These were (i) 50 mg/l streptomycin sulphate (MEA+), (ii) 50 mg/l streptomycin sulphate and 100 mg/l cycloheximide (MEA+cyclo), and (iii) 50 mg/l streptomycin sulphate, 40 mg/l benomyl and 20 mg/l dichloran (MEA+BD). Streptomycin sulphate was added to prevent the growth of bacteria, cycloheximide was used as a selective medium for fungi in the Ophiostomatales (Wingfield et al. 2022a) and benomyl-dichloran-emended media was used to select for Basidiomycete fungi (Worrall 1991). All fungi were grown in the dark at room temperature.

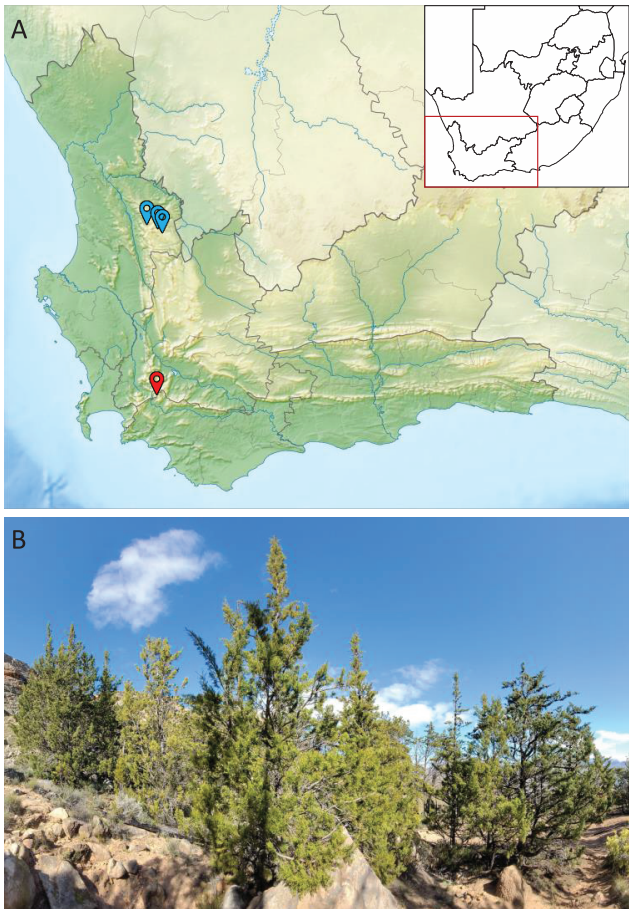

Figure 1. (A) Sampling locations of *Widdringtonia cedarbergensis* (blue) and *W. nodiflora* (red) in the Western Cape Province, South Africa. (B) *Widdringtonia cedarbergensis* at the De Rif site in the Cederberg. The map on which the locations are displayed (A) is licensed under CC BY-SA 3.0 DEED (https://creativecommons.org/licenses/by-sa/3.0/deed.en) and is available at https://en.m.wikipedia.org/wiki/File:South_Africa_Western_Cape_relief_location_map.svg.

Fungi were isolated directly from living beetles, their frass and from the walls of beetle galleries. Individual living beetles were placed in Eppendorf tubes with 0.9 ml sterile distilled water and shaken vigorously for 1 minute to loosen fungal spores on their integuments after which 0.3 ml of the solution was plated onto each selective medium. Beetle galleries in infested wood tissues were inspected for frass and fungal growth. When present, a small quantity of beetle frass (*ca*. 1 mm^3) was plated directly onto each selective medium. Fungal hyphae and spores were lifted from gallery walls using a sterile needle and plated on the same three media.

Fungal colonies were purified by transferring hyphal tips to clean agar plates. Cultures were subsequently assigned to morphological groups based on characteristics such as growth form (yeast-like or mycelial), texture, colour and pattern. For isolates with similar morphology, microscopic morphological characteristics were also examined to confirm whether they should reside in the same or different morphological groups (Kolařík et al. 2004).

Fungal morphogroups were identified by sequencing the internal transcribed spacer gene region (ITS) of representative isolates with primers ITS1F (Gardes, Bruns 1993) and ITS4 (White et al. 1990). For this purpose, actively growing fungal mycelium was harvested from each representative isolate. DNA was extracted from freshly scraped mycelia using a Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corp., USA) following the manufacturer's protocols. DNA was amplified with an initial denaturation at 94 ˚C for 3 minutes, followed by 30 cycles of 94 ˚C for 30 seconds, 54 ˚C for 30 seconds, and 72 ˚C for 1 minute, and a final elongation step at 72 ˚C for 10 minutes. BioEdit Sequence Alignment Editor (Hall 1999) was used to assess sequence quality and trim low-quality regions and correct ambiguous base calls.

Isolates were identified to genus level by comparing edited sequences to the NCBI ITS_RefSeq_Fungi nucleotide databases using command-line BLASTn 2.12.0+ (Altschul et al. 1990). In cases where the identity was uncertain, the search was repeated against the entire nucleotide database using the online BLASTn platform. To further investigate species-level classification, phylogenetic analyses were performed on the commonly occurring Exobasidiomycete, Saccharomycete, Sordariomycete and Tremellomycete isolates in the dataset. The ITS sequences of closely related type strains, as well as the type for each genus, were obtained from NCBI GenBank and ML phylogenies were constructed as described above. Sequenced isolates were deposited in the fungal culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

RESULTS

Bark beetles

Four bark beetle species in the genus *Lanurgus* were collected in this study (Figure 2). Three species were associated with the twigs, cones and stems or thick branches, respectively, of *W. cedarbergensis* trees. The fourth species was found only in *W. nodiflora* stems and no bark beetles were found in the twigs or cones of those trees. The morphology of the *W. cedarbergensis* cone beetle (*Lanurgus* sp. 2) was congruent with the description of *Lanurgus* (Jordal 2021c) and did not resemble any known species. The *W. cedarbergensis* stem and twig beetles (*Lanurgus* sp. 1 and 3) and the *W. nodiflora* stem beetle (*Lanurgus* sp. 4) had split setae suggesting their placement in the genus *Diplotrichus* (Jordal 2021a). However, dissection of the male genitalia of *Lanurgus* sp. 1 and sp. 3 indicated that these beetles also reside in *Lanurgus* (Jordal 2021b). Males of *Lanurgus* sp. 4 were not available for investigation, but due to its morphological similarity to the other species sampled in this study and the molecular data, we have treated it in *Lanurgus*. Phylogenetic analysis of the *COI* region confirmed that all four specimens represent *Lanurgus* species that form a monophyletic group with *L. beaveri* (Figure 3).

Lanurgus sp. 1 and sp. 4 were morphologically indistinguishable, but are considered different species based on locality, host association and phylogenetic analysis. Furthermore, both species are identical to the description of *D. widdringtoniae* (Jordal 2021a), but only *Lanurgus* sp. 1 is congruent with the origin of the *D. widdringtoniae* holotype. We thus conclude that *Lanurgus* sp. 1 represents *D. widdringtoniae*, which should be transferred back to *Lanurgus*.

Diplotrichus widdringtoniae constructed very short and irregular galleries in the twigs of *W. cedarbergensis*, without a recognisable pattern (Figure 4A). Galleries often extended into deeper vascular tissues, and, in some cases, smaller twigs were ringbarked. Adult beetles were abundant, but larvae were rare and only a few early instars were found. The galleries of the *W. cedarbergensis* cone beetle, *Lanurgus* sp. 2, appeared without a recognisable pattern (Figure 4B). Infested *W. cedarbergensis* cones had two to four entrance holes on the inner walls of seed scales of semi-open cones and exit holes on the outer surface of seed scales. Some cones were almost completely hollowed out by adult beetles and larvae, leaving behind resin ducts and the central axis of the cone. Larvae were free-living in a substrate mixture of sawdust and frass and were often found in co-occurrence with Anobiidae beetle larvae. Both adults and larvae of *Lanurgus* sp. 2 were found in abundance.

The *W. cedarbergensis* stem beetle, *Lanurgus* sp. 3, visited the stem sections of both *W. cedarbergensis* and *W. nodiflora* that were placed below *W. cedarbergensis* trees at three sites in the Cederberg. A clear difference in host preference was observed where very

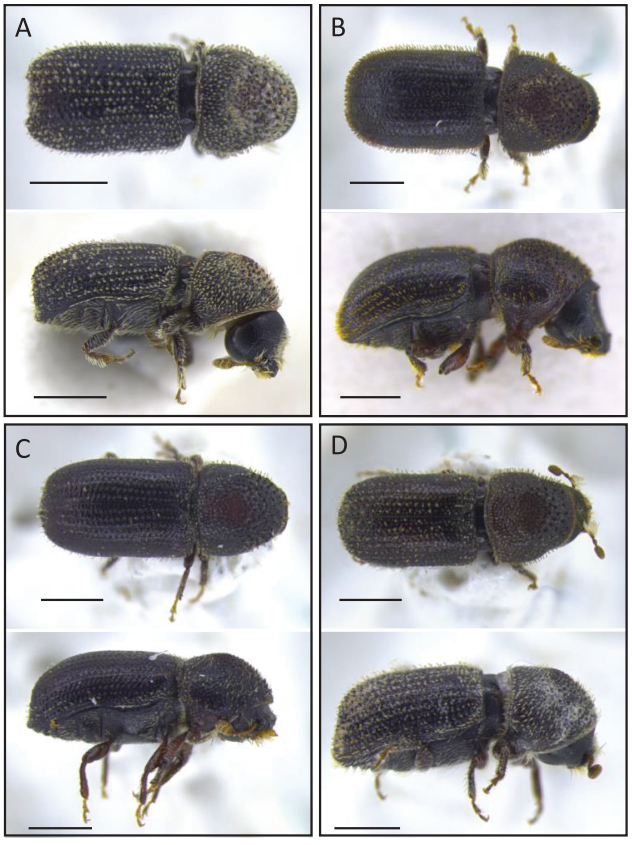

Figure 2. Dorsal (top) and lateral (bottom) views of the *Lanurgus* bark beetles collected from *Widdringtonia* trees in the Western Cape. (A) *Diplotrichus widdringtoniae* (*Lanurgus* sp. 1) from *W. cedarbergensis* twigs, (B) *Lanurgus* sp. 2 from *W. cedarbergensis* cones, (C) *Lanurgus* sp. 3 from stem sections and (D) *Lanurgus* sp. 4 from *W. nodiflora* stems. Scale bars = 500 µm.

few *Lanurgus* sp. 3 beetles entered the *W. nodiflora* stems, whereas *W. cedarbergensis* stems were infested in large numbers. In *W. cedarbergensis* stems, the beetles established an extensive network of interconnected galleries in the cambium (Figure 4C). Single isolated galleries were scarce, and did not appear to have a typical pattern, length or direction. They could be long or short and were constructed diagonally, parallel, or perpendicular with the grain of the wood. Larvae constructed galleries that were perpendicular to parental tunnels. To pupate, final instar larvae bored into the sapwood at a right angle to the surface of the cambium. In contrast, the galleries within *W. nodiflora* stems were short and most adults died without emerging. Some emergence holes were, however, present on the surface of the wax-sealed ends of the stem sections, indicating that *Lanurgus* sp. 3 can reproduce in *W. nodiflora* stems to some extent.

The *W. nodiflora* stem beetle, *Lanurgus* sp. 4, constructed long, X-shaped parental galleries in the cambium at right angles to the vascular tissue (Figure 4D). Larval galleries were perpendicular to the parental galleries and parallel with the grain of the vascular tissue. As in the case of *Lanurgus* sp. 3, final instar larvae bored into the sapwood to pupate. Even though larvae were common, adult beetles were rarely seen.

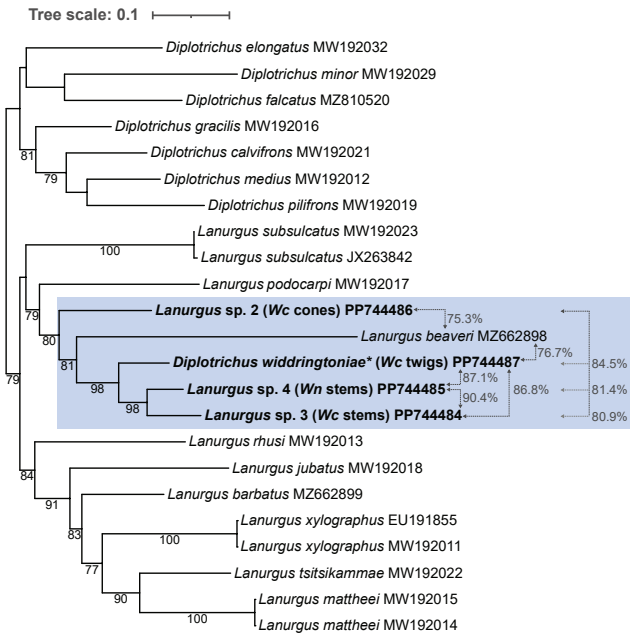

Figure 3. A maximum likelihood (ML) phylogeny of species in the genera *Lanurgus* and *Diplotrichus* based on the *COI* gene region. Taxa collected from *Widdringtonia cedarbergensis* (*Wc*) and *W. nodiflora* (*Wn*) in this study are in bold and pairwise nucleotide identity among these taxa are indicated. The asterisk highlights *Lanurgus* sp. 1 that is currently known as *D. widdringtoniae*. Reference sequences were obtained from Jordal (2021c). Branch values represent the Transfer Bootstrap Expectation (TBE) metric, based on 1000 replicates; values above 70% are shown. The final loglikelihood of the tree was –5979.646314.

Fungal associates

A total of 770 fungal isolates were obtained from the four beetle species. These were placed in 101 morphogroups, from which 40 genera could be identified based on BLASTn searches of the ITS region (Supplementary Tables S1 and S2 and Supplementary File 1). Most of these taxa were found infrequently, with only 10 genera occurring in more than 15% of the galleries or cones investigated for each beetle (Table 1 and S3). The species identity of the commonly occurring Exobasidiomycete, Saccharomycete, Sordariomycete and Tremellomycete isolates were further investigated with ML phylogenetic analysis (Table 1; Figure S1). No taxa were identified to species level, however, two yeasts (one Tremellomycete and one Saccharomycete) and a Sordariomycete species were reported as *species affinis* (*aff.*), due to a well-supported sister relationship to a single type strain, but may represent novel species. The identities of the remaining isolates could not be resolved with ITS.

A Tremellomycete (Basidiomycota) yeast, *Piskurozyma aff. capsuligenum*, was most frequently associated with *D. widdringtoniae* in *W. cedarbergensis* twigs. Four different morphogroups of this species were identified, one of which was shared with a *Cryptococcus* sp. (Figure S1A). Accounting for the shared morphogroup, *P. aff. capsuligenum* was estimated to occur in 63.6% of *Lanurgus* sp. 1 galleries, whereas other genera occurred at a frequency of 33% or less (Table 1).

The species most frequently associated with *Lanurgus* sp. 2 cone beetles, at 66.1%, was also a yeast, but of the Saccharomycetes (Ascomycota; Table 1). ITS data could not resolve the species of *Yamadazyma* but suggested that the isolates are closely related to *Y. mexicana* and *Y. luoyangensis* (Figure S1B). Four different *Yamadazyma* morphogroups were identified and one of these was shared with the second most frequently (26.8%) occurring Saccharomycete yeast, *Hyphopichia aff. pseudoburtonii*. A further

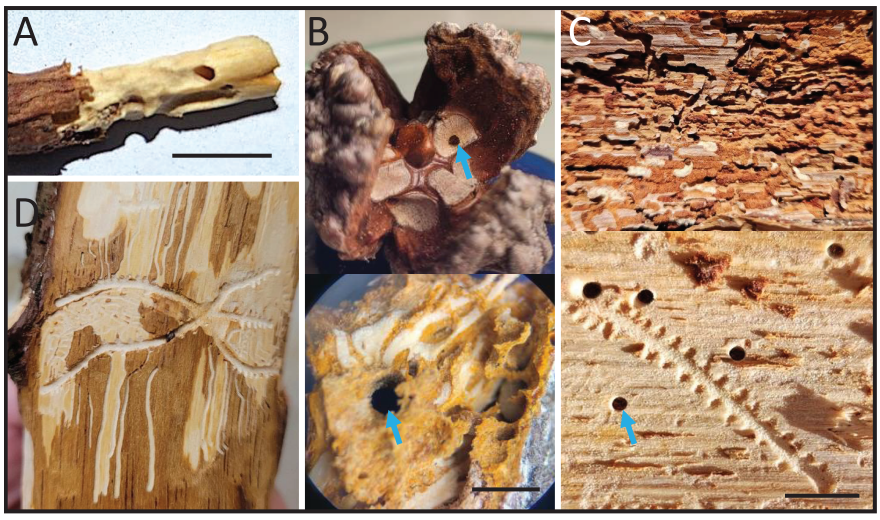

Figure 4. Galleries of the *Lanurgus* species collected in this study. (A) Gallery of *Diplotrichus widdringtoniae* (*Lanurgus* sp. 1) in a *Widdringtonia cedarbergensis* twig. (B) *W. cedarbergensis* cone with entrance hole of *Lanurgus* sp. 2 indicated with an arrow (top) and galleries inside a cone (bottom), exit hole indicated with an arrow. (C) An interconnected network of *Lanurgus* sp. 3 galleries in the inner bark of *W. cedarbergensis* (top) and gallery with pupation hole indicated with arrow (bottom). (D) X-shaped gallery of *Lanurgus* sp. 4 in *W. nodiflora*. Scale bars in A and C = 5 mm; scale bar in B = 2 mm.

Table 1. Most frequently occurring[a] fungal taxa identified from *Lanurgus* beetles in this study (see Supplementary Tables S2 and S3 for full list and details).

	Occurrence frequency of fungal taxa in beetle galleries				
Fungal species[b]	*Lanurgus* sp.1[c]	*Lanurgus* sp.2	*Lanurgus* sp.3 (Wc)[d]	*Lanurgus* sp.3 (Wn)[d]	*Lanurgus* sp.4
Exobasidiomycetes					
Quambalaria sp.			31.82%		
Dothideomycetes					
Alternaria spp.	33.33%	10.71%	6.82%		
Saccharomycetes					
Hyphopichia aff. pseudoburtonii		26.79%			
Yamadazyma sp(p).		66.07%			
Sordariomycetes					
Tolypocladium aff. pustulatum	21.21%		31.82%		
Geosmithia spp.	7.58%		42.05%	33.33%	88.24%
Sarcostroma sp.			31.82%		
Tremellomycetes					
Cryptococcus sp(p).	7.58%	21.43%			
Piskurozyma aff. capsuligenum	63.64%	3.57%	22.16%		
Teunia spp.	18.18%				

[a] Occurrence frequency was calculated as the proportion of galleries of a particular beetle from which a particular fungal taxon was isolated.
[b] The species-level identities of all taxa in this table, except the common environmental genus *Alternaria*, were investigated with phylogenetic analyses (see Figure S1).
[c] *Lanurgus* sp. 1 is currently known as *Diplotrichus widdringtoniae* (Jordal 2021a).
[d] *Lanurgus* sp. 3 beetles infesting naturally occurring *Widdringtonia cedarbergensis* (Wc) trees in the Cederberg and thick stems of *W. nodiflora* (Wn) placed in the same area artificially.

two morphogroups, occurring in 21.4% of cones, represented *Cryptococcus* sp(p) (Figure S1A).

The two stem beetles, *Lanurgus* sp. 3 from the Cederberg and *Lanurgus* sp. 4 from *W. nodiflora* on the Franschhoek Pass, were primarily associated with species of *Geosmithia* (Table 1). A strong association was particularly apparent in *Lanurgus* sp. 4, with 88.2% of galleries yielding *Geosmithia* isolates, in contrast to the 33–42% in *Lanurgus* sp. 3. ITS resolved well-supported clades of *Geosmithia* isolates from both beetle species, but these did not group with any described species (Figure S1C). The ML phylogeny suggested that at least two undescribed *Geosmithia* species were associated with each individual beetle species. A *Geosmithia* species associated with *Lanurgus* sp. 3 beetles infesting its natural *W. cedarbergensis* host was also isolated from the frass of these beetles infesting *W. nodiflora* stems artificially placed in the Cederberg. In addition to more than one *Geosmithia* taxon, more than one fungal genus was isolated from *Lanurgus* sp. 3 galleries in *W. cedarbergensis*, but not in *W. nodiflora* (Table 1).

Three other fungal taxa were associated with *Lanurgus* sp. 3 galleries in *W. cedarbergensis* at an estimated frequency of 31.8% (Table 1). These were a *Quambalaria* sp. (Exobasidiomycetes; Figure S1D) closely related to as *Q. pusilla* (syn. *Q. simpsonii*) and *Q. cyanescencs*, an unresolved or potentially novel species of *Sarcostroma* (Sordariomycetes) and *Tolypocladium aff. pustulatum* (Sordariomycetes; Figure S1C). The latter was also associated with *Lanurgus* sp. 1 at an estimated frequency of 18.2%. Other genera occurred at a frequency of 22% or less.

DISCUSSION

Four *Lanurgus* bark beetle species and their fungal associates were collected and tentatively identified in this study. Each beetle inhabited a particular part of its *Widdringtonia* host tree and was strongly associated with a specific group of yeasts or filamentous fungi. Morphological and phylogenetic analyses indicated that three of the bark beetle species represent undescribed members of the genus *Lanurgus* (tribe Micracidini; Jordal 2021b; 2021c), whereas the fourth is also a *Lanurgus* sp. that is currently known as *D. widdringtoniae*. Three of these beetles, including *D. widdringtoniae*, were collected from *W. cedarbergensis* growing naturally in the Cederberg Mountains and the final species was from a natural stand of *W. nodiflora* on the Franschhoek Pass. These bring the number of *Lanurgus* species known from the Western Cape Province to 12 (Jordal 2021b). This is the first time that bark beetles infesting *W. cedarbergensis* and *W. nodiflora* have been documented comprehensively.

Diplotrichus widdringtoniae and the *Lanurgus* sp. 2 cone beetle are the only two beetle species that have likely been recorded previously. *Diplotrichus widdringtoniae* has been collected in the Cederberg area on several occasions (Jordal 2021a; Wingfield et al. 1988), but this is the first study to show a strong link to *W. cedarbergensis* as a host and confirm that it belongs to the genus *Lanurgus*, as originally described by Schedl (1962). In a study spanning five years, Botha (1990) recorded that the cones of *W. cedarbergensis* were consistently "riddled" with *Eidophelus usagaricus* (Eggers 1922) beetles and larvae and described small round entrance holes on the cone scales that are like those observed in this study. Although herbarium material for direct comparison is not available, the study of Botha (1990) most likely represents the first discovery of *Lanurgus* sp. 2 from the present study, misidentified as *Eidophelus*.

Each *Lanurgus* species was clearly associated with a different part of its host tree, either the stems and thick branches, twigs, or cones. Niche differentiation allows different species to co-exist by lowering the degree of inter- and intraspecific competition (Økland et al. 2009) and in this case may be an example of how inter- and intraspecific competition has driven bark beetle diversification. The observation that bark beetle species infesting the same host tree often prefer different parts of the tree based on bark thickness (Amezaga, Rodríguez 1998; Paine et al. 1981), could explain the niche separation observed between beetles occupying stems and branches, namely *Lanurgus* sp. 1 that infested the twigs and *Lanurgus* sp. 3 that infested the stems and thick branches of *W. cedarbergensis*. The host preference of *Lanurgus* sp. 3 for *W. cedarbergensis* could also be observed in the lower numbers of this beetle that infested *W. nodiflora* stem sections artificially placed in the Cederberg.

Numerous fungal species were isolated from the beetles and their immediate environment, but only a few taxa emerged

as consistently associated with specific beetles. These taxa were identified based on ITS as the accepted universal fungal barcoding region (Schoch, Seifert 2012; Schoch et al. 2012). Several taxa isolated in this study appeared to represent novel lineages, however, it is important to recognise that this conclusion is based on a single barcode region. The nature and extent of the beetle-fungus relationships also remains unclear, but frequent isolation of some fungal taxa, such as *Geosmithia* species and certain yeasts, strongly suggests a symbiotic relationship, rather than an incidental association. Such symbioses typically have a nutritional benefit for the insect, either by providing nutrients directly or detoxifying defensive compounds produced by the tree host (Six 2013). Other fungal associates may provide advantages to beetles by outcompeting antagonistic fungi (Biedermann, Vega 2020; Six 2013). It is becoming increasingly apparent that fungal associates of beetles often comprise communities and not only individual species (Hulcr, Stelinski 2017), as exemplified in the present study.

Geosmithia spp. (Hypocreales) were the most common associates of both *Lanurgus* sp. 3 and *Lanurgus* sp. 4 that infested the stems and thicker branches of the two *Widdringtonia* hosts. *Geosmithia* is emerging as a common bark beetle associate and has been documented from several bark beetles on Pinaceae and Cupressaceae hosts (Kolařík, Hulcr, 2023). The rate at which *Geosmithia* species are being formally described is lagging behind the rate of novel species discovery and many *Geosmithia* species are known only as numeric identities (Kolařík, Hulcr, 2023). Further investigation of the identities of the variety of *Geosmithia* isolates obtained in this study is, therefore, needed (Aylward et al. in press). Although *Geosmithia* spp. were the predominant associates of *Lanurgus* sp. 3 in the Cederberg, other Ascomycete and Basidiomycete taxa were also present.

Three other filamentous fungi associated with *Lanurgus* sp. 3 beetles at a frequency >30% were interesting because of their close relationship to notable species. The *Quambalaria* sp. (Microstromatales) is closely related to two species that are known eucalypt associates, of which *Q. cyanescens* is a grapevine trunk pathogen (Narmani, Arzanlou 2019) and *Q. pusilla* may co-occur with plant pathogens (Chen et al. 2017). *Sarcostroma* (Xylariales) is a leaf-associated genus comprising several species that have been isolated from native South African fynbos vegetation, such as Proteacaea and Restionaceae species (Lee et al. 2006; Liu et al. 2019). The isolate sequenced in this study may represent a novel *Sarcostroma* sp. associated with an endangered South African endemic tree. Finally, several entomopathogens, parasites and endophytes occupy the genus *Tolypocladium* (Hypocreales; Gazis et al. 2014) and *T. pustulatum*, the sister taxon to the isolates in this study, is known from wood and soil (Bills et al. 2002). These potentially novel taxa may warrant further investigation from a biodiversity perspective, as well as to study the evolution of economically important species in the genera *Quambalaria* and *Tolypocladium*.

In contrast to the filamentous fungal associates of the stem beetles, yeasts were predominantly isolated from the *W. cedarbergensis* twig and cone beetles. A basidiomycete yeast species with a sister relationship to *Piskurozyma capsuligenum* was strongly associated with *D. widdringtoniae* in twigs, whereas the ascomycete *Yamadazyma* spp. were common in *Lanurgus* sp. 2 cone galleries. *Piskurozyma* species are common wood inhabitants and the genus has been associated with both beetle-infested and uninfested wood (Dighton et al. 2021). *Yamadazyma* species are common bark beetle associates, and the two species closely related to the isolates in this study are known from rotting wood (*Y. luoyangensis*) and several genera of bark beetles (*Y. mexicana*) (Chakraborty et al. 2020; Gao et al. 2021; Rivera et al. 2009).

Even though they were not strong associates, it is significant that three morphogroups of Tremellales yeasts were isolated from *D. widdringtoniae* and *Lanurgus* sp. 2. This is because these yeasts were similar to environmental *Cryptococcus* spp. that are sister to the human pathogenic *C. neoformans/C. gattii* species complex (Passer et al. 2019). South Africa is the source of two of the three recognised environmental cryptococci. These are the monotypic species *C. wingfieldii*, isolated from the frass of a scolytid beetle in Stellenbosch (Van Der Walt et al. 1987), and *C. amylolentus*, originally isolated from two Bostrichidae beetles (Passer et al. 2019). Isolation of environmental cryptococci in this study highlights a close relationship with African scolytine beetles and reveals a potential environmental source of *Cryptococcus* spp. that warrants further investigation.

Given the rich woody plant diversity of South Africa, the environmental variation, and the high diversity of xylophagous insects such as Scolytinae (Hulcr et al. 2015b; O'Brien et al. 1998), it is not surprising that this study has identified undescribed bark beetle species in a poorly studied area. All four beetle species collected would benefit from in depth morphological and phylogenetic comparisons with type material of other *Lanurgus* species and *D. widdringtoniae*. Although most fungal genera associated with these beetles are known to be involved in beetle symbioses, ITS data was sufficient to suggest that several species isolated in this study are potentially novel. Together with the *Lanurgus* beetles, the species-level identification of these fungal taxa, therefore, also deserves further study.

ACKNOWLEDGEMENTS

We are grateful to the Oppenheimer Memorial Trust (OMT) and the University of Pretoria for funding that made this study possible. Cape Nature is also acknowledged for providing the permits required for sample collection. We thank Dr A Johnson, University of Florida, and Dr BH Jordal, University of Bergen, for their assistance with examining the morphological characteristics of the beetles.

ORCID IDS

Francois Roets: https://orcid.org/0000-0003-3849-9057
Michael J. Wingfield: https://orcid.org/0000-0001-9346-2009
Janneke Aylward: https://orcid.org/0000-0002-9038-9595

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology. 215(3):403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Amezaga I, Rodríguez MÁ. 1998. Resource partitioning of four sympatric bark beetles depending on swarming dates and tree species. Forest Ecology and Management. 109(1-3):127–135. https://doi.org/10.1016/S0378-1127(98)00229-1.

Aylward J, Marincowitz S, Basson RJ, Rippon W, Wingfield MJ, Roets F. In press. Four new *Geosmithia* species from bark beetles infesting indigenous South African trees. Fungal Biology.

Biedermann PHW, Vega FE. 2020. Ecology and Evolution of Insect–Fungus Mutualisms. Annual Review of Entomology. 65(1):431–455. https://doi.org/10.1146/annurev-ento-011019-024910.

Bills GF, Polishook JD, Goetz MA, Sullivan RF, White JF. 2002. *Chaunopycnis pustulata* sp. nov., a new clavicipitalean anamorph producing metabolites that modulate potassium ion channels. Mycological Progress. 1(1):3–17. https://doi.org/10.1007/s11557-006-0001-3.

Botha S. 1990. Seed-bank dynamics in the Clanwilliam cedar (*Widdringtonia cedarbergensis*) and the implications of beetle-triggered seed release for the survival of the species. South African Journal of Ecology. 1:53–59.

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 25:1972–1973. https://doi.org/10.1093/bioinformatics/btp348.

Chakraborty A, Modlinger R, Ashraf MZ, Synek J, Schlyter F, Roy A. 2020. Core Mycobiome and Their Ecological Relevance in the Gut of Five Ips Bark Beetles (Coleoptera: Curculionidae: Scolytinae). Frontiers in Microbiology 11:568853. https://doi.org/10.3389/fmicb.2020.568853.

Chen S, Liu Q, Li G, Wingfield MJ. 2017. *Quambalaria* species associated with eucalypt diseases in southern China. Frontiers of Agricultural Science and Engineering. 4:433–447. https://doi.org/10.15302/J-FASE-2017173.

Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. 2020. ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. Molecular Biology and Evolution. 37(1):291–294. https://doi.org/10.1093/molbev/msz189.

Dighton J, Walsh E, Groben G, Zhang N. 2021. Influence of southern pine beetle on fungal communities of wood and bark decomposition of coarse woody debris in the New Jersey pine barrens. Forestry Research. 1(1):1–8. https://doi.org/10.48130/FR-2021-0017.

Eggers H. 1922. Neue Borkenkäfer (Ipidae) aus Afrika.(Nachtrag I). Entomologische Blätter. 18:163–174.

Farjon A, February E, Higgins S, Fox S, Raimondo D. 2013. *Widdringtonia cedarbergensis*. The IUCN Red List of Threatened Species. https://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T30365A2793077.en [Accessed 30 March 2022].

Farjon A, Filer D. 2013. An atlas of the world's conifers: an analysis of their distribution, biogeography, diversity and conservation status. Boston: Brill.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology. 3:294–299.

Gao W-L, Li Y, Chai C-Y, Yan Z-L, Hui F-L. 2021. New species of *Yamadazyma* from rotting wood in China. MycoKeys. 83:69–84. https://doi.org/10.3897/mycokeys.83.71156.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes -application to the identification of mycorrhizae and rusts. Molecular Ecology. 2(2):113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.

Gazis R, Skaltsas D, Chaverri P. 2014. Novel endophytic lineages of *Tolypocladium* provide new insights into the ecology and evolution of *Cordyceps*-like fungi. Mycologia. 106(6):1090–1105. https://doi.org/10.3852/13-346.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41:95–98.

Hebert PDN, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences. 270(1512):313–321. https://doi.org/10.1098/rspb.2002.2218.

Hubbard CS. 1937. Observations on the distribution and rate of growth of Clanwilliam Cedar *Widdringtonia juniperoides* Endl. South African Journal of Science. 33:572–586.

Hulcr J, Atkinson TH, Cognato AI, Jordal BH, McKenna DD. 2015a. Morphology, Taxonomy, and Phylogenetics of Bark Beetles. In: FE Vega, RW Hofstetter, editors. Bark Beetles. San Diego: Academic Press; pp. 41–84. https://doi.org/10.1016/B978-0-12-417156-5.00002-2.

Hulcr J, Atkinson TH, Cognato AI, Jordal BH, McKenna DD. 2015b. Morphology, taxonomy, and phylogenetics of bark beetles. In: FE Vega, RW Hofstetter, editors. Bark Beetles: Biology and Ecology of Native and Invasive Species. Elsevier; pp. 41–84. https://doi.org/10.1016/C2013-0-04752-4.

Hulcr J, Barnes I, De Beer ZW, Duong TA, Gazis R, Johnson AJ, Jusino MA, Kasson MT, Li Y, Lynch S, Mayers C, Musvuugwa T, Roets F, Seltmann KC, Six D, Vanderpool D, Villari C. 2020. Bark beetle mycobiome: collaboratively defined research priorities on a widespread insect-fungus symbiosis. Symbiosis. 81(2):101–113. https://doi.org/10.1007/s13199-020-00686-9.

Hulcr J, Stelinski LL. 2017. The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. Annual Review of Entomology. 62(1):285–303. https://doi.org/10.1146/annurev-ento-031616-035105.

Jordal BH. 2021a. An integrated taxonomic revision of *Diplotrichus* (Coleoptera, Scolytinae) supports a Malagasy origin and single colonisation of South Africa. Zootaxa. 5047:101–122.

Jordal BH. 2021b. The mainly South African genus *Lanurgus* revised (Coleoptera, Scolytinae). Zootaxa. 5027:87–106.

Jordal BH. 2021c. A phylogenetic and taxonomic assessment of Afrotropical Micracidini (Coleoptera, Scolytinae) reveals a strong diversifying role for Madagascar. Organisms Diversity, Evolution. 21(1):279–279. https://doi.org/10.1007/s13127-021-00488-x.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution. 30:772–780. https://doi.org/10.1093/molbev/mst010.

Kolařík M, Alena K, Pažoutivá S. 2004. Morphological and molecular characterisation of *Geosmithia putterillii, G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. Mycological research. 108(9):1053–1069. https://doi.org/10.1017/S0953756204000796.

Kolařík M, Hulcr J. 2023. *Geosmithia*—widespread and abundant but long ignored bark beetle symbionts. Mycological Progress. 22(4):32. https://doi.org/10.1007/s11557-023-01880-x.

Kolařík M, Kirkendall LR. 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). Fungal Biology. 114(8):676–689. https://doi.org/10.1016/j.funbio.2010.06.005.

Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 35(21):4453–4455. https://doi.org/10.1093/bioinformatics/btz305.

Lee S, Crous PW, Wingfield MJ. 2006. Pestalotioid fungi from *Restionaceae* in the Cape floral kingdom. Studies in Mycology. 55:175–187. https://doi.org/10.3114/sim.55.1.175.

Letunic I, Bork P. 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic acids research. 49:W293–W296. https://doi.org/10.1093/nar/gkab301.

Liu F, Bonthond G, Groenewald JZ, Cai L, Crous PW. 2019. *Sporocadaceae*, a family of coelomycetous fungi with appendage-bearing conidia. Studies in Mycology. 92(1):287–415. https://doi.org/10.1016/j.simyco.2018.11.001.

Machingambi NM, Roux J, Dreyer LL, Roets F. 2014. Bark and ambrosia beetles (Curculionidae: Scolytinae), their phoretic mites (Acari) and associated *Geosmithia* species (Ascomycota: Hypocreales) from *Virgilia* trees in South Africa. Fungal Biology. 118(5-6):472–483. https://doi.org/10.1016/j.funbio.2014.03.006.

Manders PT. 1986. An Assessment of the Current Status of the Clanwilliam Cedar (*Widdringtonia cedarbergensis*) and the Reasons for its Decline. South African Forestry Journal. 139(1):48–53. https://doi.org/10.1080/00382167.1986.9630056.

Meshram V, Sharma G, Maymon M, Protasov A, Mendel Z, Freeman S. 2022. Symbiosis and pathogenicity of *Geosmithia* and *Talaromyces* spp. associated with the cypress bark beetles *Phloeosinus* spp. and their parasitoids. Environmental Microbiology. 24(8):3369–3389. https://doi.org/10.1111/1462-2920.16016.

Musvuugwa T, De Beer ZW, Duong TA, Dreyer LL, Oberlander KC, Roets F. 2015. New species of Ophiostomatales from Scolytinae and Platypodinae beetles in the Cape Floristic Region, including the discovery of the sexual state of *Raffaelea*. Antonie Van Leeuwenhoek. 108(4):933–950. https://doi.org/10.1007/s10482-015-0547-7.

Narmani A, Arzanlou M. 2019. *Quambalaria cyanescens*, a new fungal trunk pathogen associated with grapevine decline in Iran. Crop Protection. 124:104875. https://doi.org/10.1016/j.cropro.2019.104875.

O'Brien EM, Whittaker RJ, Field R. 1998. Climate and woody plant diversity in southern Africa: relationships at species, genus and family levels. Ecography. 21(5):495–509. https://doi.org/10.1111/j.1600-0587.1998.tb00441.x.

Økland B, Skarpaas O, Kausrud K. 2009. Threshold facilitations of interacting species. Population Ecology. 51(4):513–523. https://doi.org/10.1007/s10144-009-0141-9.

Paine TD, Birch MC, Švihra P. 1981. Niche breadth and resource partitioning by four sympatric species of bark beetles (Coleoptera: Scolytidae). Oecologia. 48(1):1–6. https://doi.org/10.1007/BF00346980.

Passer AR, Coelho MA, Billmyre RB, Nowrousian M, Mittelbach M, Yurkov AM, Averette AF, Cuomo CA, Sun S, Heitman J. 2019. Genetic and Genomic Analyses Reveal Boundaries between Species Closely Related to *Cryptococcus* Pathogens. mBio. https://doi.org/10.1128/mbio.00764-19.

Pauw CA, Linder HP. 1997. Tropical African cedars (*Widdringtonia*, Cupressaceae): systematics, ecology and conservation status. Botanical Journal of the Linnean Society. 123(4):297–319. https://doi.org/10.1111/j.1095-8339.1997.tb01421.x.

Pepori AL, Kolařík M, Bettini PP, Vettraino AM, Santini A. 2015. Morphological and molecular characterisation of *Geosmithia* species on European elms. Fungal Biology. 119(11):1063–1074. https://doi.org/10.1016/j.funbio.2015.08.003.

Ploetz RC, Hulcr J, Wingfield MJ, de Beer ZW. 2013. Destructive Tree Diseases Associated with Ambrosia and Bark Beetles: Black Swan Events in Tree Pathology? Plant Disease. 97(7):856–872. https://doi.org/10.1094/PDIS-01-13-0056-FE.

Rivera FN, González E, Gómez Z, López N, Hernández-Rodríguez C, Berkov A, Zúñiga G. 2009. Gut-associated yeast in bark beetles of the genus Dendroctonus Erichson (Coleoptera: Curculionidae: Scolytinae). Biological Journal of the Linnean Society. 98(2):325–342. https://doi.org/10.1111/j.1095-8312.2009.01289.x.

Santini A, Faccoli M. 2015. Dutch elm disease and elm bark beetles: a century of association. iForest – Biogeosciences and Forestry. 8:126–134. https://doi.org/10.3832/ifor1231-008.

Schedl KE. 1962. On some African bark and timber beetles. West African Timber Borer Research Unit, Technical Bulletin. 5:57–74.

Schoch CL, Seifert KA. 2012. Reply to Kiss: Internal transcribed spacer (ITS) remains the best candidate as a universal DNA barcode marker for *Fungi* despite imperfections. Proceedings of the National Academy of Sciences. 109(27):1812–1812. https://doi.org/10.1073/pnas.1207508109.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Consortium FB. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proceedings of the National Academy of Sciences. 109(16):6241–6246. https://doi.org/10.1073/pnas.1117018109.

Six DL. 2013. The Bark Beetle Holobiont: Why Microbes Matter. Journal of Chemical Ecology. 39(7):989–1002. https://doi.org/10.1007/s10886-013-0318-8.

Smith CA. 1955. Early 19th century records of the Clanwilliam cedar (*Widdringtonia juniperoides* Endl.). Journal of the South African Forestry Association. 25(1):58–65. https://doi.org/10.1080/03759873.1955.9630756.

Tribe GD. 1991. Phenology of three exotic pine bark beetle species (Coleoptera: Scolytidae) colonising *Pinus radiata* logs in the south-western Cape Province. South African Forestry Journal. 157(1):27–31. https://doi.org/10.1080/00382167.1991.9629096.

Van der Linde JA, Six DL, De Beer WZ, Wingfield MJ, Roux J. 2016. Novel ophiostomatalean fungi from galleries of *Cyrtogenius africus* (Scolytinae) infesting dying *Euphorbia ingens*. Antonie Van Leeuwenhoek. 109(4):589–601. https://doi.org/10.1007/s10482-016-0661-1.

Van Der Walt JP, Yamada Y, Ferreira NP, Richards PDG. 1987. New basidiomycetous yeasts from Southern Africa. II. *Sterigmatomyces wingfieldii* sp. n. Antonie Van Leeuwenhoek. 53(3):137–142. https://doi.org/10.1007/BF00393841.

White JDM, Jack SL, Hoffman MT, Puttick J, Bonora D, Visser V, February EC. 2016. Collapse of an iconic conifer: long-term changes in the demography of *Widdringtonia cedarbergensis* using repeat photography. BMC Ecology. 16:53. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. 18:315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.

Wingfield BD, Wingfield MJ, Duong TA. 2022a. Molecular basis of cycloheximide resistance in the Ophiostomatales revealed. Current Genetics. 68(3-4):505–514. https://doi.org/10.1007/s00294-022-01235-1.

Wingfield MJ, Marincowitz S, Pham NQ, Roets F, Paap T, Wingfield BD, Aylward J. 2022b. Cypress canker: An important disease discovered for the first time on a native South African tree. Plant Pathology. 71(8):1735–1742. https://doi.org/10.1111/ppa.13614.

Wingfield MJ, Slippers B, Roux J, Wingfield BD. 2001. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. Bioscience. 51(2):134–140. https://doi.org/10.1641/0006-3568(2001)051[0134:WMOEFF]2.0.CO;2.

Wingfield MJ, Swart WJ. 1994. Integrated management of forest tree diseases in South Africa. Forest Ecology and Management. 65(1):11–16. https://doi.org/10.1016/0378-1127(94)90253-4.

Wingfield MJ, Von Broembsen SL, Manders PT. 1988. A preliminary assessment of the threat of diseases and pests to *Widdringtonia cedarbergensis*. South African Forestry Journal. 147(1):32–34. https://doi.org/10.1080/00382167.1988.9628968.

Worrall JJ. 1991. Media for selective isolation of hymenomycetes. Mycologia. 83(3):296–302. https://doi.org/10.1080/00275514.1991.12026013.