Forest Pathology

For. Path.
doi: 10.1111/j.1439-0329.2010.00649.x

Diplodia scrobiculata found in the southern hemisphere

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Summary

Diplodia scrobiculata, a latent pathogen of *Pinus* spp. and other conifers with a limited distribution in the United States, Mexico and southern Europe, has not been reported previously in the southern hemisphere. This is unlike its close relative *Diplodia pinea* that is found in most parts of the world where pines are native or have been introduced. During an intensive *D. pinea* survey conducted in *Pinus patula* plantations in eastern parts of South Africa, a small number of isolates atypical of *D. pinea* were found. Morphological studies and DNA sequence comparisons showed that these isolates represent *D. scrobiculata*. Microsatellite analyses suggest that the South African isolates of *D. scrobiculata* might have originated from California. Pathogenicity tests showed that some of the *D. scrobiculata* isolates were as pathogenic as those of *D. pinea* on *Pinus radiata* and *Pinus elliottii*.

1 Introduction

Diplodia pinea (Desm.) Kickx. is one of the best known pathogens of *Pinus* and other conifers worldwide. It causes significant economic losses especially in association with biotic and abiotic stresses such as hail damage, pruning wounds, drought, insect damage and extreme temperatures (Swart and Wingfield 1991; Smith et al. 2002; Blodgett and Bonello 2003). Previously, four morphotypes of *D. pinea* (A, B, C and I) were recognized (Wang et al. 1985; Palmer et al. 1987; Smith and Stanosz 1995; Hausner et al. 1999; De Wet et al. 2000; Burgess et al. 2001b). Analyses using sequences for multiple nuclear loci and polymorphic microsatellite markers led the description of the B morphotype isolates as the discrete species, *Diplodia scrobiculata* (De Wet et al. 2003). The I morphotype was found to represent *Botryosphaeria obtusa* and the C morphotype isolates were phylogenetically identical to *D. pinea*, but represent a virulent form of the pathogen known only from Indonesia (De Wet et al. 2000; Burgess et al. 2001b).

Diplodia pinea has been detected in virtually every country of the world where pines are grown as non-natives (Burgess et al. 2001a, 2004a; Burgess and Wingfield 2002). Conversely, *D. scrobiculata* has a limited distribution and host range and it is known only from Mexico, California, north-central United States and southern Europe (Morelet and Chandelier 1993; Blodgett and Stanosz 1997; Burgess et al. 2004b; Lazzizera et al. 2008; Muñoz et al. 2008). In these areas, the two species are often found together on pine and other coniferous tree species (Palmer et al. 1987). *Pinus radiata,* the predominant plantation species in many regions in the southern hemisphere, is a host of *D. scrobiculata* in its native range (Burgess et al. 2004b), as is *Pinus patula* where it is native in Mexico (Burgess et al. 2004a). Thus, it is enigmatic that *D. scrobiculata* has not moved internationally in a manner similar to *D. pinea*.

Received: 24.11.2009; accepted: 24.3.2010; editor: S. Woodward

During the course of a recent intensive sampling of *P. patula* to undertake population genetic studies of *D. pinea* in South Africa, a small number of isolates having a distinct morphology were detected. The aim of this study was to identify these isolates using morphological characteristics and DNA sequence analysis.

2 Materials and methods

2.1 Sampling and isolation

Approximately 580 samples were taken from stems, branches (asymptomatic or with die-back symptoms) and cones of *P. patula* trees in plantations occurring in the KwaZulu-Natal and Mpumalanga provinces of South Africa. The samples from mature trees and 2–5-year-old saplings were taken to the laboratory, stored at 4°C and isolations were undertaken within 2 days after sampling.

Tissue samples were surface disinfested using a modification of the method of Smith et al. (2002) by dipping them into 70% EtOH for 3–5 min followed by 3.5% NaOCl and 70% EtOH for 1 min and four washes of 1 min duration in sterile distilled water. Single conidia were removed from pycnidia produced on pine needles in agar and single spore isolates were produced as described by de Wet et al. (2003). Single conidial cultures were established on 2% malt extract agar, MEA (2% m/v Biolab malt extract and 1.5% m/v Biolab agar) and are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

2.2 Morphological and cultural characteristics

Mycelial morphology and cultural characteristics resulted in the identification of 183 isolates with morphology similar to that of *Diplodia* spp. The majority of these resembled *D. pinea*, which was the target of the survey. Six of these isolates were, however, different from the others. To determine the identity of these six isolates, the characteristics of their mycelial growth, conidial size and morphology were examined in detail.

Six isolates identified as *D. pinea* were randomly selected and the six isolates representing the unknown fungus were grown on MEA for mycelium growth comparison. From each of the isolates, about 5-mm diameter plugs were aseptically transferred from the actively growing margins of cultures to the surface of 2% MEA in 90-mm Petri dishes and incubated at 25°C with three replicate plates for each isolate. Radial colony growth was measured from the centre to the edge of the plate every day starting 3 days after initiating the growth studies and until the mycelium reached the edges of the Petri dishes. The mean cumulative growth of each isolate at each time point was calculated and these were compared with each other. As the growth rates of six *D. pinea* isolates were similar, their average was compared against those of the individual rates of the unidentified isolates.

Conidial sizes and morphology for the six isolates of the unknown fungus were examined after they had been induced to sporulate on 2% water agar (Biolab, Johannesburg, South Africa) containing sterilized pine needles and incubated in 25°C under conditions of continuous light. The lengths and widths of 10 conidia per isolate were measured and the averages were computed. The surface morphology and presence or absence of septa in the conidia was assessed using a light microscope.

2.3 DNA extraction, PCR amplification and phylogenetic analysis

Cultures of the six unknown and six *D. pinea* isolates were grown on MEA in Petri dishes for 2 weeks and mycelium scraped from the surface of the agar for DNA extraction. The

mycelium was ground in the presence of tungsten beads (3 mm; Qiagen, Hilden, Germany) in warm CTAB (*N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide) using a FastPrep FP120 homogenizer (Southern Cross Biotechnology, Cape Town, South Africa) at 5 m/s for 20 s. This maceration was repeated four times before DNA was isolated.

The internal transcribed spacer regions (ITS) of the rDNA operon were amplified using primers ITS1 (5′TCC GTA GGT GAA CCT GCG GG) and ITS4 (5′GCT GCG TTC TTC ATC GAT GC; White et al. 1990). In addition, part of the translation elongation factor (EF1-α) gene region was amplified using primers EF1-728 (5′CAT CGA GAA GTT CGA GAA GG) and EF1-986R (5′TAC TTG AAG GAA CCC TTA CC; Carbone and Kohn 1999) with polymerase chain reaction (PCR) conditions and reactions as described previously for *D. pinea* (de Wet et al. 2003). Sequencing was conducted with the same primers and reactions were run on an ABI Prism™ 3100 auto-sequencer (Applied BioSystems, Foster City, CA, USA).

The sequences for the six unknown isolates and six *D. pinea* isolates were aligned with those of known isolates of *D. pinea* and *D. scrobiculata* available on GenBank (Table 1). Alignment was done using MAFFT multiple sequence alignment for amino acid and nucleic acid (http://tim-pani.genome.ad.jp/%7emafft/server/; Katoh et al. 2002). Datasets for the aligned sequences of the ITS and EF1-α gene regions were subjected to partition homogeneity tests in paup version 4.0 (Swofford 2002) to determine whether the datasets could be combined. Characters were unweighted and unordered; gaps were treated as a fifth character (new state). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, using the tree bisection–reconnection branch-swapping option, and with the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved.

Table 1. **List of isolates used in the study.**

		GenBank accession nos	
Isolate code	Species	ITS	EF-1α
CMW7776	*D. mutila*	AY972106	DQ280420
CMW190	*D. pinea*	–	AY624251
CMW4876	*D. pinea*	AY253294	AY624252
CAP166	*D. pinea*	EU392284	EU392261
CAP168	*D. pinea*	EU392285	EU392262
CAP169	*D. pinea*	EU392286	EU392263
CMW29483[1]	*D. pinea*	HM100283	HM100274
CMW29144[1]	*D. pinea*	HM100284	HM100275
CMW29323[1]	*D. pinea*	HM100285	HM100276
CBS113423	*D. scrobiculata*	DQ458900	DQ458885
CBS109944	*D. scrobiculata*	DQ458899	DQ458884
CMW189	*D. scrobiculata*	AY253292	AY624253
CMW4900	*D. scrobiculata*	AF264907	AY624255
CMW5870	*D. scrobiculata*	–	AY625254
CMW30222[1]	*D. scrobiculata*	HM100277	HM100268
CMW30223[1]	*D. scrobiculata*	HM100278	HM100269
CMW30224[1]	*D. scrobiculata*	HM100279	HM100270
CMW30225[1]	*D. scrobiculata*	HM100280	HM100271
CMW30226[1]	*D. scrobiculata*	HM100281	HM100272
CMW30227[1]	*D. scrobiculata*	HM100282	HM100273

[1]isolated in current study.
ITS, internal transcribed spacer; EF-1α, elongation factor.

2.4 Identification based on microsatellite loci

Five previously developed microsatellite loci (SS1, SS2, SS7, SS8 and SS9) (Burgess et al. 2001b) were amplified for six isolates of the unknown fungus. All simple sequence repeat (SSR)-PCR products for the isolates were multiplexed and separated on ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The mobility of SSR products were compared with those of internal size standards (LIZ-500) and allele sizes were estimated using GeneScan 2.1 and GeneMapper 3.7 computer softwares (Applied Biosystems). The allele sizes and frequencies were compared with those for *D. scrobiculata* populations from California, Mexico and north-central America (Burgess et al. 2004b).

2.5 Pathogenicity tests

Pathogenicity tests were conducted on 18-month-old *P. radiata* and *Pinus elliottii* plants using the six unknown and six *D. pinea* isolates. Pieces of bark, approximately 25 mm^2 were removed at about 20 cm from the apices of the plants using a sterile scalpel. A plug of MEA covered with actively growing mycelium of the test fungi was placed into the stem wounds and wrapped with parafilm to protect the inoculation sites from desiccation. Three *P. radiata* and three *P. elliottii* seedlings were used for each isolate inoculated and an equal number of plants were inoculated with sterile MEA plugs to serve as controls.

Lesion lengths were measured 2 weeks after inoculation. The mean lesion lengths associated with the six *D. pinea* isolates and the six isolates of the unknown fungus were analysed using anova in GenStat Discovery Edition 3 (Rothamsted Experimental Station, Hemel Hempstead, UK). Isolations were made from the lesions on MEA to determine whether the lesions had resulted from the inoculated fungi and identifications of the re-isolated fungi were made based on morphology.

3 Results

3.1 Sampling and isolation

The majority of the 183 isolates obtained during this study that resembled *Diplodia* spp. originated from the branch samples, and 14 isolates were from asymptomatic *P. patula* stems. Of the six isolates with a morphology differing from the others, four were from samples collected in a plantation in Sabie (Mpumalanga Province), and the remaining two were from the Balgown and Boston plantations (KwaZulu-Natal Province). Two of the six isolates having a distinct morphology were isolated from the internal parts of the stems of adult asymptomatic trees and the others were from branches of 3–4-year-old *P. patula* trees showing symptoms of die-back.

3.2 Morphological comparisons

The six isolates of the unknown fungus grew more slowly than those known to represent *D. pinea*. After 6 days, all colonies of the *D. pinea* isolates had abundant aerial mycelium and had covered the surface of the Petri dishes, whereas those of the unknown fungus took up to 8 days to cover the surface of the plates and the mycelium was appressed to the surface of the medium (Fig. 1). The conidia of the unknown fungus were brownish in colour, with up to three septa and pitted surfaces. The dimensions of these conidia ranged between 34.0–(36.4)–39.6 μm in length and 11.1–(13.8)–16.6 μm in width (averages in brackets).

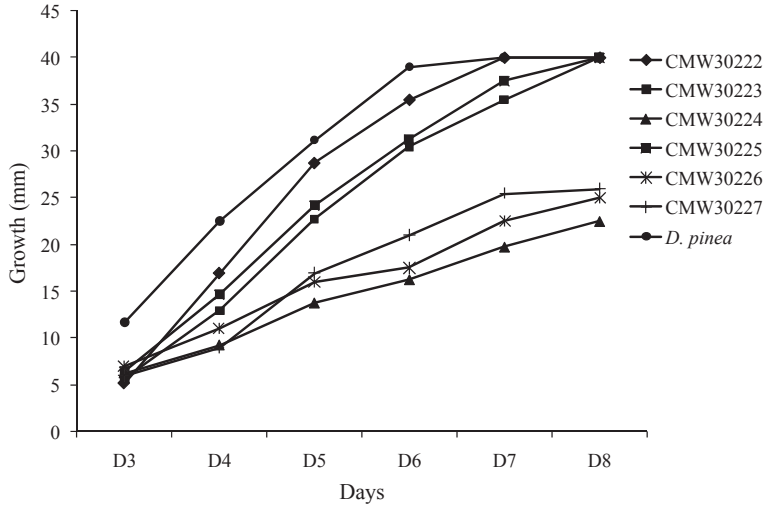

Fig. 1. Comparisons of mycelial growth of six isolates of the unknown *Diplodia* species with the average growth for six *Diplodia pinea* isolates (●) at 25°C for 8 days commencing on day three. Each growth curve for the isolates of the unknown *Diplodia* sp. represents an average of three measurements. Measurements were taken from the centre of isolates to the edge of the plate.

3.3 Phylogenetic analyses

BLAST analysis showed that the sequences for the six unidentified isolates were similar to NCBI reference *D. scrobiculata* collections with a sequence similarity of 98–99%. Moreover, the combined analysis of ITS and EF-1α DNA sequences indicated that these six isolates clustered together with sequences of *D. scrobiculata* (Fig. 2). The other isolates clustered with *D. pinea* (Fig. 2).

3.4 Microsatellite marker analyses

Of the five SSR loci, SS1 and SS2 were monomorphic and the remaining three were polymorphic for the six isolates of the unknown fungus. The polymorphic loci had two to three alleles per locus to give a total of nine alleles across the five loci. Five of these alleles were identical to alleles previously found to be unique in Californian populations of *D. scrobiculata* at loci SS1, SS2, SS7 and SS8. One allele from locus SS8 was the same as that in populations of *D. scrobiculata* from California and Mexico. The two alleles at locus SS9 from the South African population have not been observed previously in any other population (Table 2).

3.5 Pathogenicity tests

All the *D. pinea* and the six isolates identified as *D. scrobiculata* resulted in lesions that were significantly larger than those of the control treatment (Fig. 3). ANOVA showed that the lesions associated with the *D. pinea* isolates (41.51 ± 1.74 mm) were significantly longer ($p < 0.05$) than the lesions associated with *D. scrobiculata* (31.54 ± 1.7 mm) regardless of the pine species inoculated. However, some of the *D. scrobiculata* isolates (e.g. CMW30223) produced lesions that were equal in length to those associated with *D. pinea* on both pine species (Fig. 3a,b). *Diplodia scrobiculata* isolate CMW30227 produced the

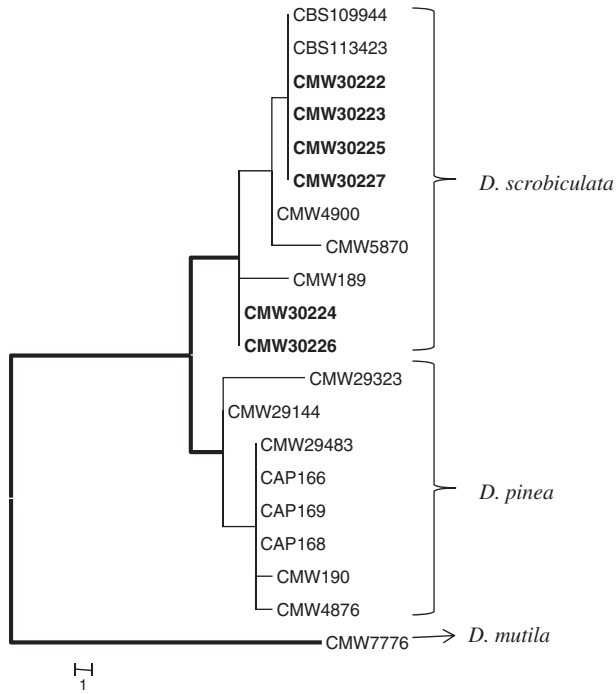

Fig. 2. Phylogenetic tree constructed from the combined internal transcribed spacer rDNA and elongation factor (EF-1α) sequences for six isolates of an unknown *Diplodia* sp. presented in bold. Bootstrap values for *D. scrobiculata* and *D. pinea* branch points are 86 and 75 respectively.

smallest lesions on both the *Pinus* spp. On *P. radiata*, lesions caused by *D. scrobiculata* varied significantly between the different isolates, but there were no significant differences between the lesions caused by *D. pinea* isolates on this pine species. *Diplodia pinea* inoculated onto *P. radiata* resulted in longer lesions than those on *P. elliottii* (Fig. 3a,b). Lesions produced by both *D. scrobiculata* and *D. pinea* varied on *P. elliottii* (Fig. 3b) indicating that this *Pinus* sp. is more tolerant to infection than *P. radiata*.

4 Discussion

Morphology and DNA sequences in this study provided robust evidence that *D. scrobiculata* is present in South Africa. This is the first time that the fungus has been found in plantations of non-native pines in the southern hemisphere. The discovery is intriguing given the fact that the closely related fungus *D. pinea* is one of the most common fungi found on pines in this area (Smith et al. 2000).

Only 1% of isolates originating from the *D. pinea* survey were found to represent *D. scrobiculata* implying that the fungus is very rare and apparently a poor competitor in the South African environment. *Diplodia pinea,* which is generally more competitive in northern hemisphere (Burgess et al. 2004b), could have already dominated the niche in South Africa before the arrival of *D. scrobiculata*. This is likely, because *Pinus* spp. were originally introduced from Europe where *D. pinea* is the dominant species (Burgess et al. 2004a). The faster growth and superior competitive ability of *D. pinea* could favour its ability to compete for resources as compared with its close relative, *D. scrobiculata*.

Table 2. **Comparisons of allele sizes (bp) and frequencies of South African (RSA) *Diplodia scrobiculata* isolates with those of populations from California (Cal), Mexico (Mex) and north-central America (NCA) at five loci. Data for Cal, Mex and NCA were taken from Burgess et al. (2004b). Shaded region indicated that alleles of the RSA population correspond to those in other populations. Unique alleles in the RSA population are shaded and in bold.**

Locus	Allele	Cal	Mex	NCA	RSA
SS1	326	–	0.050	0.050	–
	361	–	0.250	0.950	–
	342	–	0.050	–	–
	444	0.055	–	–	1.000
	468	0.945	0.250	–	–
	508	–	0.400	–	–
SS2	200	0.222	–	–	1.000
	204	–	0.250	–	–
	206	0.778	0.750	1.000	–
SS7	383	0.667	–	–	0.833
	387	0.056	–	–	–
	394	0.277	–	–	0.167
	396	–	0.050	–	–
	401	–	–	0.650	–
	404	–	–	0.300	–
	409	–	–	0.050	–
	411	–	0.500	–	–
	415	–	0.250	–	–
	419	–	0.125	–	–
	423	–	0.050	–	–
SS8	279	–	–	–	0.167
	283	0.111	–	–	0.500
	288	0.111	0.850	–	0.333
	293	–	0.100	0.150	–
	295	–	–	0.500	–
	298	–	–	0.100	–
	301	–	–	0.050	–
	305	–	0.050	0.100	–
	317	0.556	–	–	–
	322	0.056	–	–	–
	333	0.167	–	–	–
SS9	236	1.000	–	0.050	–
	237	–	–	0.950	–
	238	–	1.000	–	–
	242	–	–	–	**0.833**
	254	–	–	–	**0.167**

The rare occurrence of *D. scrobiculata* might explain why the fungus has not been found in previous studies on *D. pinea* in South Africa, Australia and New Zealand (Smith et al. 2000; Burgess et al. 2001a, 2004a). Peripherally, *D. scrobiculata* and *D. pinea* have a very similar morphology and they were treated as the same fungus for many years. Thus, isolates of *D. scrobiculata* in past studies in the southern hemisphere might also have been overlooked and treated as those of *D. pinea*.

Allele sizes generated from five SSR microsatellite markers suggest that the isolates of *D. scrobiculata* from South Africa are more similar to isolates from California, and different from those from Mexico and the north-central United States. The isolates from California that were most similar to South African isolates were isolated from the Cambria area (Burgess et al. 2004b) from which *P. radiata* germplasm was imported in 1959 to Europe, Australia and New Zealand (Burgess and Wingfield 2002). It is also known that

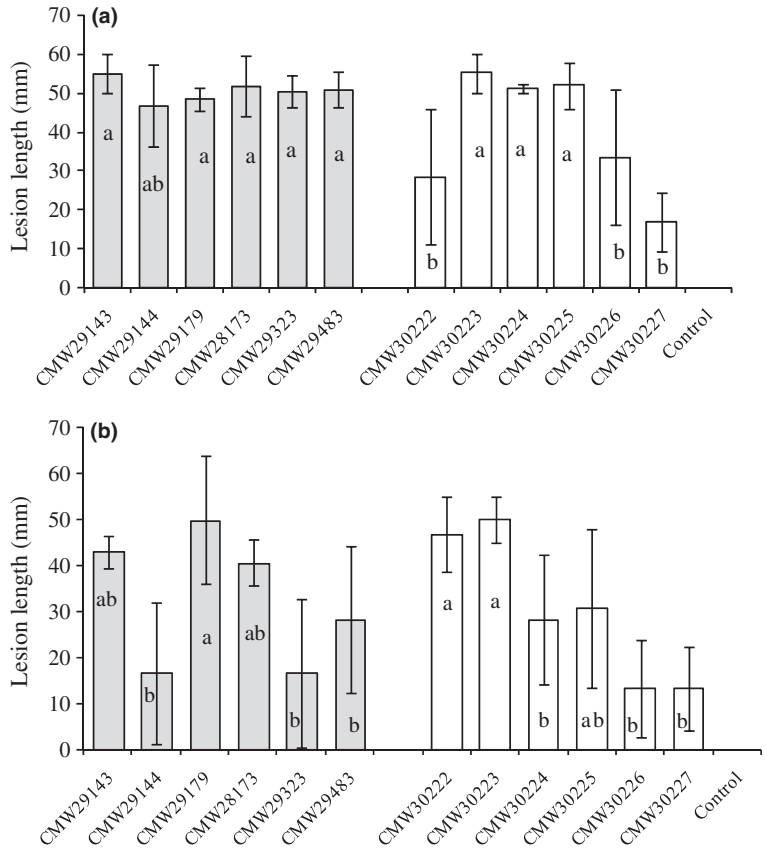

Fig. 3. Mean lesion length (mm) resulting from inoculations with six isolates for six isolates of *Diplodia pinea* (shaded) and the unknown *Diplodia* sp. (unshaded) on: (a) *Pinus radiata* and (b) *Pinus elliottii* seedlings. Vertical bars show ± SE and the same letters within a graph or *Pinus* spp. indicated that there were no significant lesion length differences at $p \leq 0.05$.

P. radiata seed has been imported into South Africa from California, which could explain the common alleles in isolates from the two locations.

Diplodia scrobiculata is a pine pathogen although pathogenicity studies have shown it to be less virulent than *D. pinea* (Palmer et al. 1987; Blodgett and Stanosz 1997, 1999; de Wet et al. 2000). Interestingly, this study suggested that it may be possible that some isolates of the former fungus are as virulent as those of *D. pinea.* Difference in virulence, however, depends on the methods of inoculation and *Pinus* spp. For example, when these two fungi were inoculated onto shoots of *Pinus banksiana* they resulted in lesions of the same size but *D. pinea* was more virulent on *Pinus resinosa* (Palmer et al. 1987). This work and previous studies have also shown that there are differences in the virulence of strains of these two fungi and further studies should be undertaken to better understand their relative virulence.

This study represents the first report of *D. scrobiculata* from the southern hemisphere. This pathogen was most probably introduced into South Africa with germplasm of *P. radiata* from California. *Diplodia scrobiculata* appears to compete less successfully with *D. pinea* and is present in South Africa at a much lower frequency than *D. pinea*. However,

contrary to previous reports, some of the genotypes of *D. scrobiculata* present in South Africa may be as virulent as those of *D. pinea* on pine species grown in the country, and as with *D. pinea,* care should be taken to avoid importation of additional genotypes of this pathogen.

Acknowledgements

The authors thank the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), members of the Tree Protection Co-operative Program (TPCP) and National Research Foundation (NRF) for funding. This research was also supported by the International Foundation for Science, Stockholm, Sweden, through a grant to Mr Wubetu Bihon.

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