Patterns of Multiple Virus Infections in the Conifer Pathogenic Fungi, *Diplodia pinea* and *Diplodia scrobiculata*

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Abstract

Diplodia pinea and *Diplodia scrobiculata* are opportunistic pathogens associated with various disease symptoms on conifers that most importantly include die-back and stem cankers. Two viruses with dsRNA genomes, *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) are found in *D. pinea* and an undescribed dsRNA element is known to occur in *D. scrobiculata*. We have partially characterized the putative RNA-dependent RNA polymerase (RdRp) of the undescribed dsRNA element and designed virus-specific primers from the RdRp regions of all three virus genomes. This made it possible to screen for the presence of the three viruses in a collection of *D. pinea* and *D. scrobiculata* isolates using real-time PCR. Triple infections with all three viruses occurred in *D. pinea* and *D. scrobiculata*. Co-infections with SsRV1 and SsRV2 were common but found only in *D. pinea*. Co-infection with SsRV1 and the undescribed dsRNA element was rare and observed only in *D. pinea*. Single infections with either SsRV1 or SsRV2 were equally common, while the undescribed dsRNA element never occurred alone. SsRV1 occurred alone in both *D. pinea* and *D. scrobiculata* while SsRV2 occurred alone only in *D. pinea*. There were only two instances where the undescribed dsRNA element was observed in *D. pinea* and it was otherwise found only in *D. scrobiculata*. This study highlights the complex interactions between the viruses found in the closely related plant pathogenic fungi, *D. pinea* and *D. scrobiculata*. It illustrates the importance of not only characterizing viruses infecting fungi but also of determining the interactions between mycoviruses and their fungal hosts.

Introduction

Diplodia pinea (Desm.) Kickx [=*Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton and *Diplodia scrobiculata* J. De Wet, Slippers and M.J. Wingf., previously known as the B morphotype of *D. pinea* (Palmer et al., 1987; De Wet et al., 2003)], are opportunistic pathogens on conifers (Eldridge, 1961; Punithalingam and Waterston, 1970; Swart et al., 1985). Both fungi are endophytes that can exist in healthy asymptomatic trees and the onset of disease is typically associated with stress (Smith et al., 1996; Stanosz et al., 1997; Flowers et al., 2001, 2003). In association with unfavourable environmental conditions or harsh physical factors, they commonly cause disease symptoms including die-back, whorl cankers and seedling collar rot (Eldridge, 1961; Gibson, 1979; Swart and Wingfield, 1991). *Diplodia pinea* is an important pathogen of *Pinus* spp. in natural forests and plantations of non-native species, in many parts of the world (Punithalingam and Waterston, 1970;Swart et al., 1985; Stanosz et al., 1999; Burgess et al., 2001). In contrast, *D. scrobiculata* has a more limited distribution known only from the North Central USA and Western Europe and it tends to be weakly pathogenic (Palmer et al., 1987; Blodgett and Stanosz, 1997; Stanosz et al., 1999; Burgess et al., 2004).

Diseases caused by *D. pinea* and *D. scrobiculata* are managed through the exploitation of resistant host species and the implementation of optimal management strategies and silvicultural practices (Swart et al., 1985; Swart and Wingfield, 1991). Significant economic losses due to *D. pinea* infections are, however, incurred, especially in plantations of non-native pine species in the southern hemisphere (Gibson, 1979; Zwolinski et al., 1990a,b). An alternative to conventional control might be found in the application of hypovirulence-mediated mycoviruses as biocontrol agents (Anagnostakis, 1982; Heiniger and Rigling, 1994). Various studies have, therefore, considered whether dsRNA elements occur in *D. pinea* (Wu et al., 1989; Preisig et al., 1998; Steenkamp et al., 1998; De Wet et al., 2001; Adams et al., 2002).

Several dsRNA elements ranging from 600 bp to 7 kb in size have been reported from *Diplodia* isolates (Wu et al., 1989; Preisig et al., 1998; Steenkamp et al., 1998; De Wet et al., 2001; Adams et al., 2002). Two of these elements have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig et al., 1998). A third dsRNA element associated with *D. scrobiculata* is known but it has not been characterized (De Wet et al., 2001). SsRV1 and SsRV2 belong to the family *Totiviridae* and are treated as unclassified *Totiviridae*. They are characterized by unipartite dsRNA genomes with two open reading frames, one coding for a capsid polypeptide and the other one for a RNA-dependant RNA polymerase (RdRp). SsRV1 and SsRV2 genomes are in the 5 kb size range, while the undescribed dsRNA element in *D. scrobiculata* is slightly larger. The dsRNA elements associated with *D. pinea* and *D. scrobiculata* do not appear to result in phenotypic changes to the pathogen or to reduce its virulence (Steenkamp et al., 1998; De Wet et al., 2001). Despite the fact that these viruses have no apparent phenotypic effect, the presence of specific viruses in their host populations serve as a useful marker in studying movement of fungal pathogens.

Multiple infections with different cytoplasmic dsRNA elements, as well as, mitochondrial dsRNA elements are common in fungi (Buck, 1986). For example, in a single *Helminthosporium victoriae* isolate, two viruses were found, one belonging to the *Totiviridae* (Huang and Ghabrial, 1996) and the other to the *Chrysoviridae* (Ghabrial et al., 2002). Likewise, three different viruses a totivirus (*G. abietina* RNA virus L2 or GaRV-L2), a partitivirus (*G. abietina* RNA virus MS2 or GaRV-MS2) and a mitovirus (*G. abietina* mitochondrial RNA virus S2 or GaMRV-S2) have been found in a single *Gremmeniella abietina* var. *abietina* type A isolate (Tuomivirta and Hantula, 2005). Thus, the discovery of two different totiviruses, SsRV1 and SsRV2, in a single *D. pinea* isolate (Preisig et al., 1998) was not unusual.

In a previous study, using SsRV1- and SsRV2-specific primers, it was shown that some isolates are infected by either SsRV1 or SsRV2, or a combination of the two viruses (De Wet et al., 2001). The ability to detect these genomes in infected strains is inconsistent as the titre of the viruses can be low and variable depending on the culture conditions of the fungus. Traditionally, detection of specific viral genomes has depended on the use of blotting techniques, which have a limited sensitivity. Real-time PCR is increasingly being used for virus detection, especially in the medical field (Mackay et al., 2002). This technology is not only extremely sensitive but it also allows for relatively accurate and rapid quantification of the concentration of the viral genomes.

Diplodia pinea and *D. scrobiculata* are closely related fungi that have only recently been recognized as distinct. Thus, an intriguing question relates to the relative distribution of the three viruses in isolates of the two fungi. Consequently, this study was undertaken to screen a collection of *D. pinea* and *D. scrobiculata* isolates from various parts of the world for the presence of SsRV1, SsRV2 and the undescribed virus element known only from *D. scrobiculata*. To achieve this goal, we partially characterized a putative RdRp of the undescribed dsRNA element associated with *D. scrobiculata*. The full genome sequence is consequently being determined and phylogenetically assessed in comparison with other mycoviruses.

Materials and Methods

DsRNA extraction, cDNA synthesis and cloning of a putative RdRp gene

A single conidial isolate (CMW5870) of *D. scrobiculata* from California was grown in 2 ml Eppendorf tubes containing 2% malt extract broth (Biolab Diagnostics, Midrand, South Africa), incubated at room temperature for at least 2 weeks. Mycelium was harvested by centrifugation and washed with 0.1% diethylpyrocarbonate (DEPC)-treated double-distilled water. The mycelium was homogenized using a Retsch MM301 homogenizer (Laboratories (Pty) Ltd., Parkhurst, South Africa 30 freq/s; 30 s). Trizol (Invitrogen Corporation, Carlsbad, CA, USA) was used to extract dsRNA from the mycelium (1 ml Trizol per 0.5 g mycelium). After centrifugation, the supernatant was passed through a QIAquick gel extraction column (QIAGEN, GmbH, Germany) following the manufacturer's specifications. The eluted sample was separated on a 1% agarose (w/v) gel (Biolab Diagnostics, Midrand, South Africa) stained with ethidium bromide, using a

1× Tris–Acetic Acid–EDTA (pH 8) electrophoresis buffer. The dsRNA band was cut from the gel and purified using the QIAquick gel extraction kit (QIAGEN).

Synthesis of cDNA from dsRNA was performed using a Roche cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). The dsRNA and random hexamer primers were subjected to denaturing conditions (10 min at 99 °C), which was followed by the first and second strand cDNA syntheses done as specified by the manufacturer. The synthesized cDNA was purified using a QIAquick gel extraction kit (QIAGEN) and cloned using the Lucigen PCR-SMART non-proofreader cloning kit (Lucigen® Corporation, Middleton, WI, USA). A colony PCR was performed using CL3 and SR2 pcrSMART™ vector-specific primers. Colony PCR products with inserts were purified using the Roche PCR product purification kit (Roche Diagnostics) and sequenced.

Genome-specific primers were designed from the random amplified cDNA fragments to amplify larger pieces of the putative RdRp using the Roche Titan One Tube RT-PCR system (Roche Diagnostics). Single band RT-PCR products were purified using the Roche PCR product purification kit and sequenced. Non-specific RT-PCR products were gel purified using the PCR product purification kit (Roche Diagnostics) and cloned using the pGEM®-T Easy Vector System II (Promega Corporation, Madison, WI, USA). A colony PCR was performed using T7 and SP6 pGEM®-T Easy vector-specific primers. Colony PCR products with inserts were purified using the Roche PCR product purification kit (Roche Diagnostics) and sequenced.

Sequencing was achieved using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster, CA, USA). All reactions were done using protocols recommended by the manufacturers. All the sequence data were processed using Chromas 2.3 (http://www.technelysium.com.au) and contigs were assembled using Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Contigs were aligned in BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA).

Primer development

Virus-specific primers for SsRV1 and SsRV2 were designed from the open reading frame (ORF) coding for the RdRp of these viruses (Preisig et al., 1998) The full-length virus genomes are available on GenBank (SsRV1: AF038665; SsRV2: AF039080) (http://www.ncbi.nlm.nih.gov/). The SsRV1-specific primers are SsRV1-F1 (5'-GACGGCCCCGTCTACAACACAGAC-3') and SsRV1-R1 (5'-GGGCGGCGCGTTCCACCTCCGAC-3') (1951–2102). The SsRV2-specific primers are SsRV2-F1 (5'-GCCGTTGCGCCCAACCGCTCGAGG-3') and SsRV2-R2 (5'-GGTCTGCGCCTCACTGGGCCGAGG-3') (2033–2183). The sequence of the putative RdRp ORF of the undescribed dsRNA element associated with *D. scrobiculata* as determined in this study was deposited in GenBank (EF568774). Primers specific to this undescribed dsRNA element were designed and these primers are DsRV1-F2 (5'-GGTATCGCTGGTTACCCGATCCGC-3') and DsRV1-R2 (5'-CAGATGGGGCTCAAAGGCACCTCC-3') (1781–1934).

Fungal isolates used for genotyping

A total of 32 *D. pinea* and *D. scrobiculata* isolates from South Africa, North Central United States, Mexico, Madagascar, Colombia and California were used in this study (Table 1). The identity of these isolates had been determined in previous studies (Stanosz et al., 1999; De Wet et al., 2000, 2003; Adams et al., 2002). Single conidial cultures of these isolates were obtained from the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 1 *Diplodia pinea* and *Diplodia scrobiculata* isolates used in this study, as well as *T*m-values obtained after real-time PCR for the three distinct viruses

					***T*m-values[b]**		
Isolates[a]	**Species**	**Origin**	**Host**	**Collector**	**SsRV 1**	**SsRV 2**	**Undescribed dsRNA**
CMW4254*	*D. pinea*	Gauteng, South Africa	*P. roxburghii*	MJ Wingfield	88.85	87.26	No product
CMW4241	*D. pinea*	Western Cape, South Africa	*P. radiata*	MJ Wingfield	89.03	86.15	No product
CMW5848	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.02	87.37	No product
CMW5849	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	90.29	87.70	No product
CMW5850	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.10	87.51	No product
CMW5852	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.61	No product	No product
CMW5853	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	No product	88.00	No product
CMW5854	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.45	87.37	No product
CMW5855*	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	90.19	87.50	No product
CMW5856	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	88.9	89.29	No product
CMW5857*	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.86	87.99	No product
CMW5858*	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	No product	87.46	No product
CMW5859	*D. pinea*	Colombia	*P. patula*	MJ Wingfield	89.87	88.43	No product
CMW5860*	*D. pinea*	Madagascar	*P. patula*	O Preisig	89.53	87.73	87.14

CMW5861*	*D. pinea*	Madagascar	*P. patula*	O Preisig	88.85	86.91	No product
CMW5862	*D. pinea*	Madagascar	*P. patula*	O Preisig	89.08	No product	No product
CMW5863	*D. pinea*	Madagascar	*P. patula*	O Preisig	88.50	No product	No product
CMW5864	*D. pinea*	Madagascar	*P. patula*	O Preisig	88.54	No product	87.27
CMW5865	*D. pinea*	Madagascar	*P. patula*	O Preisig	90.02	No product	No product
CMW25385	*D. pinea*	Unknown	*P. ponderosa*	GC Adams	No product	88.42	No product
CMW25386	*D. pinea*	Unknown	*P. radiata*	GC Adams	No product	87.94	No product
CMW25387	*D. pinea*	MN, USA	*P. resinosa*	GC Adams	No product	88.91	No product
CMW25388*	*D. pinea*	MN, USA	*P. resinosa*	GC Adams	88.99	87.73	No product
CMW12513	*D. pinea*	France	*Larix* sp.	P Chandelier	No product	89.06	No product
CMW13234	*D. pinea*	France	*Cedrus* sp.	P Chandelier	89.04	88	No product
CMW4900	*D. scrobiculata*	Mexico	*P. greggii*	MJ Wingfield	89.31	No product	No product
CMW5867	*D. scrobiculata*	Mexico	*P. greggii*	MJ Wingfield	89.10	87.35	87.41
CMW5868	*D. scrobiculata*	Mexico	*P. patula*	MJ Wingfield	No product	No product	No product
CMW5869	*D. scrobiculata*	CA, USA	*P. radiata*	MJ Wingfield	89.18	No product	No product
CMW5870	*D. scrobiculata*	CA, USA	*P. radiata*	MJ Wingfield	89.30	88.30	87.69
CMW5871	*D. scrobiculata*	CA, USA	*P. radiata*	MJ Wingfield	88.91	87.68	87.01
CMW5847*	*D. scrobiculata*	MI, USA	*P. banksiana*	M Palmer	89.02	86.77	87.95

[a]CMW refers to the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] *T*m-values are the melting points of each product.

No product means that no virus was detected.

Isolates for which virus-specific products were sequenced are marked with an asterisk (*).

Total RNA isolations

Single conidial cultures were transferred to 2% malt extract agar (Biolab Diagnostics) and incubated at 25°C under cool white light. After 4 days, mycelium was scraped from the borders of the cultures and transferred to 500 μl 2% malt extract broth (Biolab

Diagnostics) in 2 ml Eppendorf tubes. These were incubated at 25°C for at least 2 weeks or until sufficient biomass had been produced for total nucleic acid isolations. Mycelium was harvested by centrifugation and washed with DEPC-treated double-distilled water. The mycelium was homogenized using a Retsch MM301 homogenizer (30 freq/s; 30 s). Trizol (Invitrogen) was used to extract total RNA from the homogenized mycelium (1 ml Trizol per 500 mg mycelium). The suspension was incubated at room temperature for 5 min, 200 μl chloroform was added, vigorously shaken and incubated a second time at room temperature for 5 min. The supernatant containing total RNA was recovered through centrifugation at 14 463 ***g*** at 4°C for 10 min. Supernatant was passed through a QIAquick gel extraction column (QIAGEN) according to the manufacturer's specifications. Samples were stored at −20°C.

cDNA synthesis and real-time PCR genotyping

Synthesis of cDNA from the extracted total RNA was performed using the Roche Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). The total RNA and virus-specific primers was firstly denatured for 5 min at 99°C followed by the first strand cDNA synthesis done according to the manufacturer's instructions. For each isolate, cDNA was synthesized using all three anti-sense primers, separately.

Real-time amplification was achieved using the LightCycler® 480 SYBR Green I Master (Roche Diagnostics). A one in 10 dilution was made of the cDNA and 5 μl was added to 10 μl SYBR Green Master Mix and each specific primer pair to a final concentration of 0.5 μM (final volume = 20 μl). Amplification was carried out in a 384-well plate in the LightCycler® 480 Real Time PCR system (Roche Diagnostics) using an initial denaturation of 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 62°C for 5 s and 72°C for 8 s. Fluorescence was recorded at the annealing step for each cycle. The amplification cycles were followed by a melting cycle, in which DNA was denatured at 95°C for 30 s, cooled to 50°C using a rate of 1°C/s and held for 30 s. Temperature was then raised to 95°C with a transition rate of 10 acquisitions/°C. Fluorescence was continuously monitored during the melting cycle. This was followed by a cooling cycle to 40°C for 20 s.

Melting curves were converted into negative derivative curves of fluorescence with respect to temperature (-dF/dT) by the LightCycler® Data Analysis software. These showed whether a sequence-specific product with a unique melting temperature (Tm) had been obtained. Non-specific amplification products such as primer dimers could be distinguished from sequence-specific products based on their lower melting points. The final amplification products were electrophoresed on a 1.5% agarose gel to confirm that the melting curve analysis reflects the amplicons of *ca.* 150 bp.

Amplicon sequence confirmation

The identity of a sub-set of amplicons was confirmed by sequencing (Table 1). A one in 10 dilution of the amplified product was made and 5 μl was used in a 25-μl reaction mixture consisted of 10× Fast Start PCR buffer [50 mM Tris–HCl, pH 8.3; 2 mM $MgCl_2$; 10 mM

KCl; 5 mM $(NH_4)_2SO_4$], 200 μM of each dNTP, 0.2 μM of each primer and 2 U FastStart Taq DNA polymerase (Roche Diagnostics). The following temperature profile was followed: 95° C for 5 min, 45 cycles of 95° C for 10 s, 62° C for 5 s and 72° C for 8 s, followed by a final elongation at 72° C for 5 min. PCR products were electrophoresed on a 1.5% agarose gel. Products without primer dimers were purified using the Roche High Pure PCR Product Purification kit (Roche Diagnostics), and those with primer dimers were gel purified using the same kit. Purified products were then sequenced using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems). All the reactions were done using protocols recommended by the manufacturers. The sequence data were processed in Chromas 2.3 (http://www.technelysium.com.au) and aligned in Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). DNA sequences of fragments obtained were subjected to BLAST using the NCBI-translated database (Blastx) to confirm their identities. Sequences obtained for SsRV1 and SsRV2 were aligned to the original genome sequences. Sequences obtained for the undescribed dsRNA element associated with *D. scrobiculata* were aligned to the putative RdRp to confirm similarity, as well as, to determine whether any differences were present.

Results

Partial characterization of a putative RdRp gene

DsRNA was extracted from a Californian isolate of *D. scrobiculata* (CMW5870). DNA fragments of various sizes were obtained after cDNA synthesis with random hexamer primers. Theses sequences were subjected to BLAST searches using the NCBI-translated database (Blastx). Sequences with homology to the RdRp of *Trichomonas vaginalis* virus II (TVV2) were retained. These fragments were aligned according to the RdRp of TVV2 and specific primers were designed to amplify a total of 2174 bp. The nucleotide sequence that was obtained translated to 724 amino acids containing all eight conserved motifs as described by Bruenn (1993). The obtained RdRp gene for the undescribed virus associated with *D. scrobiculata* has 24% homology to the RdRp of TVV2, 24% homology to the RdRp of SsRV1 and 27% homology to the RdRp of GaRV-L1. The sequence of the putative RdRp associated with *D. scrobiculata* was deposited in GenBank (EF568774), while the rest of the genome is being determined.

cDNA synthesis and real-time PCR genotyping

Total RNA was extracted from 32 *D. pinea* and *D. scrobiculata* isolates collected from a wide range of geographic locations (Table 1). cDNA was then synthesized and amplified for all the isolates. Melting curve analyses revealed three unique melting points for SsRV1, SsRV2 and the undescribed dsRNA element associated with *D. scrobiculata*. The *T*m for SsRV1 ranged from 88.5 to 90.3°C that for SsRV2 from 86.2 to 89.1°C and the *T*m for the dsRNA element associated with *D. scrobiculata* ranged from 87.0 to 87.7°C. Multiplexing was not possible as the *T*m-values of the three viruses overlapped. Agarose gel electrophoresis revealed the desired 150 bp amplicon and where primer dimers were observed, these correlated with the lower *T*m values (81–84°C) that were detected with the

melting curve analyses.

Amplicon sequence confirmation
Sequences of a sub-set of amplicons gave a 100% confirmation with the respective GenBank sequences of SsRV1, SsRV2 and the putative RdRp of the undescribed dsRNA element. A representative sample of amplicons was sequenced for SsRV1 and SsRV2 and all six amplicons of the undescribed dsRNA element were sequenced. Single base pair differences were in some case observed between the sequenced amplicons and the original genome sequence probably as a result of amplification errors. Sequence data did, however, provide confidence in the *T*m-values of each specific virus as detected by real-time PCR.

Virus distribution in isolates
All three dsRNA elements (SsRV1, SsRV2 and the undescribed dsRNA element in *D. scrobiculata*) occurred in five of the 32 isolates (16%) included in this study (Table 1). Of these five isolates, only one was of *D. pinea* and the other four were *D. scrobiculata*. SsRV1 and SsRV2 occurred together in 13 of the 32 isolates (41%) (Table 1). All of these isolates were of *D. pinea*. Single infections with only SsRV1 occurred in six of the 32 isolates (19%) and six isolates contained only SsRV2. Isolates infected with only SsRV1 were of *D. pinea* and *D. scrobiculata*. Isolates infected only with SsRV2 were all of *D. pinea*. One *D. pinea* isolate contained both SsRV1 and the undescribed dsRNA element. One *D. scrobiculata* isolate was not infected with any of the dsRNA elements.

Discussion

This study is the first to consider the presence of dsRNA elements in a relatively large panel of *D. pinea* and *D. scrobiculata* isolates from different parts of the world including South Africa, North Central United States, Mexico, Madagascar, Colombia and California. Results were obtained through highly reliable real-time PCR and revealed intriguing patterns of mixtures of the three different dsRNA elements, which have never previously been considered in conjunction. Triple infections were less frequent than double infections with SsRV1 and SsRV2. Single infections with either SsRV1 or SsRV2 were equally common while the undescribed dsRNA element never occurred alone.

The dsRNA element in *D. scrobiculata* has not been fully characterized but it nevertheless was possible to sequence the putative RdRp of this dsRNA element. This putative RdRp has relative low homology to the RdRps of SsRV1 and SsRV2, as well as, those of other members of the *Totiviridae* namely *G. abietina* RNA virus L1 (GaRV-L1) and *Trichomonas vaginalis* virus 2 (TVV2). The generic position of the undescribed dsRNA element must await sequencing of its complete genome, but the relatively low homology to RdRps of members of the *Totiviridae* might be an indication that this dsRNA element is represented by another virus family. This would not be unusual as most previous studies suggest a polyphyletic origin for fungal viruses (Koonin et al., 1989; Ahn and Lee, 2001; Tuomivirta and Hantula, 2005).

An interesting result emerging from this study was that SsRV1 and SsRV2 were detected in both *D. pinea* and *D. scrobiculata* isolates. These two viruses were first discovered in a South African isolate of *D. pinea* (Preisig et al., 1998). They have never previously been found in *D. scrobiculata*. The latter fungus is relatively closely related to *D. pinea* (De Wet et al., 2003) and for many years was known as the B morphotype of that fungus (Palmer et al., 1987). The two fungi are quite different, based on ecology, morphology and phylogenetic inference (Wang et al., 1985; Palmer et al., 1987; De Wet et al., 2000, 2003). However, results of this study, showing that they share infections with SsRV1 and SsRV2, support the fact that they are closely related and the view that they probably evolved concurrently. The undescribed dsRNA element was almost always found in isolates of *D. scrobiculata* but there were two intriguing exceptions. These were for two isolates of *D. pinea* from Madagascar. A broader survey is, however, needed to determine whether *D. pinea* and *D. scrobiculata* share infections with the undescribed dsRNA element.

This is the first report of triple infections with SsRV1, SsRV2 and the undescribed dsRNA element in *D. pinea* and *D. scrobiculata*. Co-infections with SsRV1 and SsRV2 were more frequently observed than triple infections. SsRV1 and SsRV2 have previously been shown to co-infect *D. pinea* (Preisig et al., 1998), but results of this study showed co-infections with SsRV1 and SsRV2 occurred in both *D. pinea* and *D. scrobiculata*. Single infections with either SsRV1 or SsRV2 were equally likely in both *D. pinea* and *D. scrobiculata*. Single infections with only the undescribed dsRNA element were never observed. The undescribed dsRNA element in *D. scrobiculata* is not a defective segment of SsRV1 and SsRV2 as its RdRp is vastly different to those of the SsRVs, but it could be dependent on the presence of SsRV1 and SsRV2. The sample size considered in this study was, however, insufficient to determine whether this might be the case. Multiple infections with viruses from the same and different families have previously been reported. In the canker pathogen of conifers, *G. abietina* three viruses belonging to three different virus families namely *Totiviridae*, *Partitiviridae* and *Mitovirus* were sequenced (Tuomivirta and Hantula, 2005). Likewise, in the Dutch elm fungus, *Ophiostoma novo-ulmi* four mitoviruses have been sequenced (Hong et al., 1998). The role of these multiple infections and the interactions between the different viruses and their fungal hosts is still unknown. This study has illustrated the complexity of the interactions between the three viruses associated with *D. pinea* and *D. scrobiculata*. These viruses also appear to be non-host specific and are easily transmitted between their different fungal hosts. This can be indicative of a more ancient origin where the viruses adapted to survive in more than one host over time.
The effects of different dsRNA elements, singly or as multiple infections, on their fungal hosts is relatively unexplored. Previous studies (Steenkamp et al., 1998; De Wet et al., 2001) have shown no phenotypic characteristics linked to the presence of dsRNA elements in *D. pinea* or *D. scrobiculata*. In contrast, Adams et al. (2002) observed that dsRNA-cured *D. pinea* cultures are sometimes more virulent than their dsRNA-containing parental cultures in 1 year and less virulent the following year. Future studies will thus concentrate on fully characterizing the dsRNA element commonly found in *D. scrobiculata* and we will then consider the possible effect of these infections on the biology of the two host fungi.

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