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## CHAPTER 5

# PATTERNS OF MULTIPLE VIRUS INFECTIONS IN THE CONIFER PATHOGENIC FUNGI, *DIPLODIA PINEA* AND *DIPLODIA SCROBICULATA*

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## ABSTRACT

*Diplodia pinea* and *D. 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*. In this study, we 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.

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## INTRODUCTION

*Diplodia pinea* (Desm.) Kickx (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton and *D. scrobiculata* J. de Wet, Slippers & 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 & 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 & 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 & 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 & 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 & 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, 1990b). An alternative to conventional control might be found in the application of hypovirulence-mediated mycoviruses as biocontrol agents (Anagnostakis 1982; Heiniger & 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 – 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 & 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 &

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*.

## **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 (ME) broth (Biolab Diagnostics, Midrand, South Africa), incubated at room temperature for at least two 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 (30 freq/s; 30 s). Trizol (Invitrogen Corporation, Carlsbad, CA, USA) was used to extract dsRNA from the mycelium (1ml 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 x Tris-Acetic Acid-EDTA (TAE) (pH 8) electrophoresis buffer. The dsRNA band was cut from the gel and purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany).

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, GmbH, Germany) 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, Basel, Switzerland) 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, Basel, Switzerland). 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, Basel, Switzerland) and cloned using

the pGEM<sup>®</sup>-T Easy Vector System II (Promega Corporation, Madison, WI, USA). A colony PCR was performed using T7 and SP6 pGEM<sup>®</sup>-T Easy vector-specific primers. Colony PCR products with inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and sequenced.

Sequencing was achieved using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, 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, Carlsberg, 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 *Diplodia 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; Adams *et al.* 2002; De Wet *et al.* 2003). 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.

### **Total RNA isolations**

Single conidial cultures were transferred to 2 % malt extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) and incubated at 25 °C under cool white light. After four days, mycelium was scraped from the borders of the cultures and transferred to 500 µl 2 % malt extract (ME) broth (Biolab Diagnostics, Midrand, South Africa) in 2 ml Eppendorf tubes. These were incubated at 25 °C for at least two 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 Corporation, Carlsbad, CA, USA) was used to extract total RNA from the homogenized mycelium (1ml 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 12 000 rpm (14 463 x g) at 4 °C for 10 min. Supernatant was passed through a QIAquick gel extraction column (QIAGEN, GmbH, Germany) 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, Basel, Switzerland). 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, Basel, Switzerland). A one in ten 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, Basel, Switzerland) 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 (T<sub>m</sub>) 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 ten dilution of the amplified product was made and 5 µl was used in a 25 µl reaction mixture consisted of 10 x Fast Start PCR buffer (50 mM Tris-HCl, pH 8.3; 2 mM MgCl<sub>2</sub>; 10 mM KCl; 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 200 µM of each dNTP, 0.2 µM of each primer and 2 U FastStart Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland). 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, Basel, Switzerland), and those with primer dimers were gel purified using the same kit. Purified products were then sequenced using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). 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. These 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 – 90.3 °C, that for SsRV2 from 86.2 - 89.1 °C and the  $T_m$  for the dsRNA element associated with *D. scrobiculata* ranged from 87.0 – 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) 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 & Lee 2001; Tuomivirta & 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 & 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 one 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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**Table 1.** *Diplodia pinea* and *D. scrobiculata* isolates used in this study, as well as T<sub>m</sub>-values obtained after Real-Time PCR for the three distinct viruses.

Isolates <sup>a/</sup>	Species	Origin	Host	Collector	T <sub>m</sub> – values <sup>b/</sup>		
					SsRV1	SsRV2	Undescribed dsRNA
CMW4254*	<i>D. pinea</i>	Gauteng, South Africa	<i>P. roxburghii</i>	MJ Wingfield	88.85	87.26	No product
CMW4241	"	Western Cape, South Africa	<i>P. radiata</i>	"	89.03	86.15	No product
CMW5848	"	Colombia	<i>P. patula</i>	"	89.02	87.37	No product
CMW5849	"	"	"	"	90.29	87.70	No product
CMW5850	"	"	"	"	89.10	87.51	No product
CMW5852	"	"	"	"	89.61	No product	No product
CMW5853	"	"	"	"	No product	88.00	No product
CMW5854	"	"	"	"	89.45	87.37	No product
CMW5855*	"	"	"	"	90.19	87.50	No product
CMW5856	"	"	<i>P. patula</i>	"	88.9	89.29	No product
CMW5857*	"	"	"	"	89.86	87.99	No product
CMW5858*	"	"	"	"	No product	87.46	No product
CMW5859	"	"	"	"	89.87	88.43	No product
CMW5860*	"	Madagascar	<i>P. patula</i>	O Preisig	89.53	87.73	87.14
CMW5861*	"	"	"	"	88.85	86.91	No product
CMW5862	"	"	"	"	89.08	No product	No product
CMW5863	"	"	"	"	88.50	No product	No product
CMW5864	"	"	"	"	88.54	No product	87.27
CMW5865	"	"	"	"	90.02	No product	No product
CMW25385	"	Unknown	<i>P. ponderosa</i>	GC Adams	No product	88.42	No product
CMW25386	"	Unknown	<i>P. radiata</i>	"	No product	87.94	No product
CMW25387	"	Minnesota, USA	<i>P. resinosa</i>	"	No product	88.91	No product
CMW25388*	"	"	"	"	88.99	87.73	No product
CMW12513	"	France	<i>Larix</i> sp.	P Chandelier	No product	89.06	No product
CMW13234	<i>D. pinea</i>	France	<i>Cedrus</i> sp.	P Chandelier	89.04	88	No product



Isolates <sup>a/</sup>	Species	Origin	Host	Collector	Tm – values <sup>b/</sup>		
					SsRV1	SsRV2	Undescribed dsRNA
CMW4900	<i>D. scrobiculata</i>	Mexico	<i>P. greggii</i>	MJ Wingfield	89.31	No product	No product
CMW5867	"	"	"	"	89.10	87.35	87.41
CMW5868	"	"	<i>P. patula</i>	"	No product	No product	No product
CMW5869	"	California, USA	<i>P. radiata</i>	"	89.18	No product	No product
CMW5870	"	"	"	"	89.30	88.30	87.69
CMW5871	"	"	"	"	88.91	87.68	87.01
CMW5847*	"	Michigan, USA	<i>P. banksiana</i>	M Palmer	89.02	86.77	87.95

<sup>a/</sup>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. <sup>b/</sup>Tm-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 \*.



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**CHAPTER 6**

**CHARACTERIZATION OF A NOVEL dsRNA ELEMENT IN  
THE PINE ENDOPHYTIC FUNGUS, *DIPLODIA*  
*SCROBICULATA***

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## ABSTRACT

*Diplodia scrobiculata* and *Diplodia pinea* are endophytic fungi associated with die-back and cankers of mainly *Pinus* spp. in many parts of the world. These two fungi are closely related and have in the past been considered to represent two morphological forms (A and B morphotypes) of *D. pinea*. DsRNA elements are known to occur in both *D. scrobiculata* and *D. pinea*. Two dsRNA elements from *D. pinea*, SsRV1 and SsRV2 have previously been characterized. The aim of this study was to characterize a third dsRNA element that is most commonly associated with *D. scrobiculata* and to determine its phylogenetic relationship with other mycoviruses. The 5018 bp genome of this element was sequenced and it is referred to as *D. scrobiculata* RNA virus 1 or DsRV1. It has two open reading frames (ORFs) one of which codes for a putative polypeptide with a high homology to proteins of the vacuolar protein-sorting (VPS) machinery and the other for a RNA dependent RNA polymerase (RdRp). Phylogenetic comparisons based on the amino acid alignments of the RdRp revealed that DsRV1 is closely related to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2), and they grouped separately from virus families in which mycoviruses have previously been described. Although *D. pinea* and *D. scrobiculata* are closely related, DsRV1 does not share a high sequence homology with SsRV1 or SsRV2 and they probably have different evolutionary origins.

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## INTRODUCTION

*Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. is a weak, opportunistic pathogen of mainly *Pinus* spp. that co-exists with the well-known pine pathogen, *D. pinea* (Desm.) Kickx, where their host ranges overlap (Palmer *et al.* 1987; Burgess *et al.* 2004b). This fungus was previously known as the B morphotype of *D. pinea* (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2003). Disease symptoms commonly associated with *D. pinea* and *D. scrobiculata*, in combination with various stress-inducing environmental or physical factors include die-back, cankers, collar rot and a root disease (Punithalingham & Waterston 1970; Wingfield & Knox-Davies 1980; Swart & Wingfield 1991).

*Diplodia scrobiculata* and *D. pinea* can be distinguished based on morphology, distribution, virulence and DNA sequence comparisons (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2000, 2002, 2003). *Diplodia scrobiculata* has a low level of virulence and a restricted distribution, while *D. pinea* can be highly virulent and it has a world-wide distribution (Blodgett & Stanosz 1997; Burgess & Wingfield 2002; Burgess *et al.* 2004a, 2004b). The genetic structure of *D. scrobiculata* populations compared to those of *D. pinea* is also different. Populations of *D. scrobiculata* are geographically isolated, with little gene flow, high allelic diversities and no multilocus genotypes shared between populations. These factors suggest a recent history of recombination and/or mutation (Burgess *et al.* 2004a). Populations of *D. pinea* show indications of a long asexual history with moderate to low gene diversities and multilocus genotypes that are shared between populations (Burgess *et al.* 2004b).

Several dsRNA elements of different size have been reported from *D. pinea* and *D. scrobiculata* (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002). Two of these, isolated from a South African A morphotype *D. pinea* isolate, have been



characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig *et al.* 1998). They are characterized by monopartite dsRNA genomes in the 5 kb size range with two ORFs. One of these ORFs codes for a capsid polypeptide (CP) and the other for a RdRp. Based on these characteristics and phylogenetic relationships, they have been shown to be closely related to viruses in the genus *Totivirus*, family *Totiviridae* (Preisig *et al.* 1998). In a recent study, a third dsRNA element was isolated from a Californian *D. scrobiculata* isolate (De Wet *et al.* 2008).

Multiple infections with different viruses are common in fungi (Buck 1986). The frequency and distribution of the three viruses associated with *D. pinea* and *D. scrobiculata* was determined using Real-time PCR with virus-specific primers (De Wet *et al.* 2008). SsRV1 and SsRV2 were found to occur in both *D. pinea* and *D. scrobiculata*, while the third dsRNA element was mainly associated with *D. scrobiculata* isolates except for two *D. pinea* isolates from Madagascar.

Interestingly, the third dsRNA element was found never to occur alone but always in combination with SsRV1 and/or SsRV2. The occurrence of multiple infections with three different viruses in these two closely related fungal species highlights the complex dynamics of the viral populations associated with *D. scrobiculata* and *D. pinea*.

Most mycoviruses are latent, causing no visible effects on their fungal hosts (Buck 1986; Ghabrial 1998). Initial studies on the dsRNA elements associated with *D. pinea* and *D. scrobiculata* showed that they have no significant effect on the virulence of these fungi (Steenkamp *et al.* 1998; De Wet *et al.* 2001). However, in a study conducted by Adams *et al.* (2002), a dsRNA-containing *D. pinea* isolate was found to be significantly less virulent than its dsRNA-free sub-culture, therefore, showing the potential of being able to attenuate virulence.

The aim of this study was to determine the sequence of the third dsRNA element associated with *D. scrobiculata*, which we refer to as *Diplodia scrobiculata* RNA virus 1 (DsRV1). A further aim was to use phylogenetic comparisons to determine the relatedness of DsRV1 to other fungal viruses.

## **MATERIALS AND METHODS**

### **Fungal isolate and dsRNA extraction**

A single conidial *D. scrobiculata* isolate (CMW5870) from California was used in this study and it is maintained in the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, as well as the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. The fungus was grown in 250 ml Erlenmeyer flasks containing 2 % malt extract (ME) broth (Biolab Diagnostics, Midrand, South Africa), incubated at 25 °C with shaking (150 rpm) for at least two weeks or until sufficient biomass was produced for dsRNA extraction. Mycelium was harvested by centrifugation and then lyophilized. The lyophilized mycelium was ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. Trizol (Invitrogen Corporations, Carlsbad, CA, USA) and chloroform was used to extract dsRNA from the mycelium (1ml Trizol per 0.5 g mycelium). The supernatant obtained after centrifugation at 12 000 rpm at 4 °C for 10 minutes was precipitated overnight with 0.7 volumes isopropanol and 0.1 volumes sodium acetate. The dsRNA was recovered through centrifugation for 30 min at 13 000 rpm at 4 °C, washed with 70 % ethanol, dried and re-suspended in 50 µl DEPC (0.1% diethylpyrocarbonate)-treated dH<sub>2</sub>O. The isolated dsRNA was separated on a 1 % agarose (w/v) gel (Biolab Diagnostics, Midrand, South Africa) stained with ethidium bromide, using a 1 x Tris-acetic acid-EDTA (TAE) (pH 8) electrophoresis buffer. The largest dsRNA fragment (Fig. 1) was cut from the gel using a non-

UV transilluminator (DarkReader). The excised dsRNA fragment was purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany), treated with RNase free DNase I for 2 hours at 37 °C and stored at -20 °C until further use.

### **Synthesis and cloning of cDNA using random hexamer primers**

Synthesis of cDNA from dsRNA was performed using the Roche cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). The dsRNA and random hexamer primers were denatured for 10 min at 99 °C followed by the first and second strand syntheses done following the manufacturer's instructions. The synthesized dsDNA was purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany) and cloned using the Lucigen PCR-SMART non-proofreader cloning kit (Lucigen® Corporation, Middleton, WI, USA). Ligated plasmids were transformed into *E. coli*® chemically competent cells (Lucigen® Corporation, Middleton, WI, USA) and transformants were grown on YT-medium supplemented with kanamycin (final concentration of 30 µg/ml). A colony PCR was performed using forward CL3 and reverse SR2 primers specific to the pcrSMART™ vector. The 25 µl reaction mixture consisted of 1x PCR buffer (50 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 8), 200 µM of each dNTP, 0.2 µM of each primer and 0.25 U Fast start *Taq* polymerase (Roche Diagnostics, Basel, Switzerland). The following temperature profile was followed: 6 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C, and a final elongation step for 7 min at 72 °C. Colony PCR products were visualised on a 1 % agarose gel containing ethidium bromide using UV illumination. PCR amplified inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and sequenced.

### **Amplification and cloning of the complete viral genome**

The random amplified cDNA fragments were aligned according to the RdRp gene of the *Trichomonas vaginalis* virus 2 (TVV2), as they shared homology and genome-specific primers were designed. Sequences between the cDNA fragments were obtained through RT-PCR with the genome-specific primers using the Roche Titan One Tube RT-PCR system (Roche Diagnostics, Basel, Switzerland). The 50 µl reaction mixture containing 1x RT-PCR buffer (1.5 mM MgCl<sub>2</sub> and DMSO), 5 mM DTT, 0.2 mM each dNTP, 5 U RNase Inhibitor, 1 µl enzyme mix, 0.4 µM each primer and the dsRNA template. The primers and dsRNA were firstly denatured for 10 min at 99 °C and cooled on ice. The rest of the reaction mix was then added to the denatured dsRNA followed by reverse transcription for 30 minutes at 50 °C. This was followed by PCR amplification of 1 cycle at 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min, 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min with a cycle elongation of 5 s per cycle and finally an elongation step of 10 min at 68 °C.

RT-PCR products were visualised on 1 % agarose gels containing ethidium bromide using UV illumination. Single band cDNA 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, Basel, Switzerland) and ligated overnight to the pGEM-T Easy Vector System II (Promega Corporation, Madison, WI, USA). Ligated plasmids were transformed into *Escherichia coli* JM109 cells (Promega Corporation, Madison, WI, USA) and screened for transformants on LB-medium supplemented with X-Gal (Fermentas Life Sciences, Lithuania) and IPTG (Fermentas Life Sciences, Lithuania). Colony PCR, as described in the previous section, was performed using T7 and SP6 primers. PCR amplified inserts were purified using the Roche PCR product purification kit and sequenced.

### **Determination of the distal ends of the viral genome**

TAIL-PCR (thermal asymmetric interlaced) (Liu & Whittier 1995; Nakayama *et al.*, 2001) and RLM-RACE (RNA ligase-mediated amplification of cDNA ends) (Coutts & Livieratos 2003) were used to obtain the distal ends of the viral genome. TAIL PCR entailed three consecutive PCR reactions using TAIL-cycling between high-stringency and low-stringency cycles using three nested genome-specific primers and eight degenerate primers. RLM-RACE was based on the ligation of an oligonucleotide (PC4: GCATTCGACCCGGGTT) to the dsRNA using T4 RNA ligase (Roche Diagnostics, Basel, Switzerland). This oligonucleotide was phosphorylated at the 5' end and blocked at the 3' end to prevent concatenation. First strand cDNA was then synthesized using a primer (PC5: AACCCGGGTCGTATGC) complementary to PC4 with the Fermentas First strand cDNA synthesis kit (Fermentas Life Sciences, Lithuania). This was followed by amplification of the cDNA using genome-specific primers and PC5. Products obtained were cloned using the pGEM-T Easy Vector System II, PCR amplified inserts were purified using the Roche PCR product purification kit as described previously and sequenced.

### **Isolation and amplification of genomic DNA**

The same single conidial *D. scrobiculata* isolate (CMW5870) from California from which dsRNA was extracted was grown in liquid ME medium in 1.5 ml Eppendorf tube, for one week at 25 °C. After centrifugation, the mycelial pellet was homogenized using a Retsch MM301 homogenizer (30 freq/s; 30 s), followed by the extraction of DNA using the technique described by Raeder & Broda (1985). The DNA was stored at -20 °C until further use.

ORF1- and ORF2-specific primers were tested on genomic DNA (Fig. 2a). These primers were RDF23 (5'-CCCTAACCTGCGACCTCCGTCG-3') (nt. 164) and RDR28 (5'-CCGCCATTTCTGGGGAAAGGCC-3') (nt. 1226) for ORF1 and RDF11 (5'-

CCCCGGTAGGAACGAGGTCTTCGC-3') (nt. 2180) and RDR2 (5'-CGATACCGTGCATACCGTAGAACT-3') (nt. 3309) for ORF2. As positive controls, the internally transcribed spacer (ITS) regions 1 and 2, and the 5.8S ribosomal subunit (White *et al.* 1990) and dilutions of RT-PCR products obtained from the dsRNA with the same primers, were amplified. The 25 µl reaction mixture consisted of 10x PCR buffer (50 mM Tris-HCl, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM KCl, 2 mM MgCl<sub>2</sub>, pH 8.3), 200 µM of each dNTP, 200 nM of each primer, 5 ng template and 0.1 U FastStart *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland). The following temperature profile was followed: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 52 °C and 2 min at 72°C followed by a final elongation step of 7 min at 72 °C.

PCR products were visualised on a 1% agarose gel containing ethidium bromide using UV illumination. The PCR products were then purified using the Roche High Pure PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and both DNA strands were sequenced.

### **Sequencing and sequence analysis**

All sequencing was done using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Reactions were done using protocols recommended by the manufacturers. Sequence data were processed using Chromas version 2.3 (<http://www.technelysium.com.au>) and contigs assembled using Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Alignments of overlapping contigs were done in BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsberg, CA, USA).

## Phylogenetic analysis

Translated amino acid sequences of the RdRp gene of DsRV1 were compared with 31 viruses belonging to the *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* or *Endornavirus* (Table 1). These represent virus families in which dsRNA mycoviruses have been reported. A positive sense ssRNA virus belonging to the *Potyviridae* was used as the outgroup for the comparisons. Amino acid sequences were aligned using MAFFT version 5 (Katoh *et al.* 2005). A parsimony analysis based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm was performed and bootstrap support was determined in PAUP\* after 1000 replications. A phylogram was constructed that was rooted and edited in TreeView 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

## RESULTS

### Synthesis and sequencing of cDNA from *D. scrobiculata* dsRNA

DsRNA extracted from *D. scrobiculata* mycelium and separated by gel electrophoresis revealed four segments of *ca.* 5.2 kb, 5 kb, 2 kb and 1 kb (Fig. 1). The double stranded nature of the RNA was verified by heat treatment of the native dsRNA at 99 °C to produce single stranded RNA. Amplification, using the primer pair DsRV1-F2 (5'-GGTATCGCTGGTTACCCGATCCGC-3') (nt. 3306) and DsRV1-R2 (5'-CAGATGGGGCTCAAAGGCACCTCC-3') (nt. 3458) (Fig. 2a), showed that the three smaller dsRNA fragments are deletion mutants of the largest fragment (Fig. 2b).

DNA fragments of different sizes were obtained after cDNA synthesis using denatured dsRNA of fragment 1 and random hexamer primers. These fragments were cloned and sequenced. Initial BLAST searches using the NCBI translated database (Blastx) showed homology to the RdRp of *Trichomonas vaginalis* virus 2 (TVV2).

## Genome organization of DsRV1

A total of 5018 nucleotides were assembled by overlapping contigs that were aligned according to the RdRp gene of TVV2. The complete DsRV1 sequence was cloned and sequenced at least four times to accurately determine the nucleotide positions (Genbank accession number EU547739). The dsRNA genome of DsRV1 has a GC content of 59 % and consists of two ORFs in the +3 translation frame (Fig. 2a). The existence of the two ORFs on the same dsRNA fragment was verified through RT-PCR amplification across ORFs using primers RDF20 (5'-GGAGATCACTTCGCTGTACC-3') (nt. 689) and RDR23 (5'-GGCAGCAGCCGCCTCCACGG-3') (nt. 1913) (Fig. 2a).

The first ORF (nt. 30 - 1280) encodes a putative polypeptide of 416 amino acids with a predicted molecular mass of 47.2 kDA. This polypeptide has a 60 % identity and 67 % similarity to protein complexes of the class E vacuolar protein-sorting (VPS) machinery (Q0U6X7) (Fig. 3). The context of the first methionine of DsRV1 was less favoured for translation (Kozak 1991) as it has a pyrimidine at position -3 and a purine in position +1 (CGUAUGG). It did, however, align with the amino acid sequence of a VPS protein, suggesting that it is the likely start codon of ORF1 (Fig. 3).

The second ORF (nt. 1500 – 4832) translates to 1110 amino acids coding for a RdRp with a predicted molecular mass of 122.9 kDA. It has 36 % identity and 51 % similarity to the RdRp gene of *Phlebiopsis gigantea* mycovirus 2 (Pgv2) (CAJ34335), a 25 % identity and 36 % similarity to the RdRp gene of *Sphaeropsis sapinea* RNA virus 1 (SsRV1) (NP047558) and a 24 % identity and 38 % similarity to the RdRp gene of *Trichomonas vaginalis* virus 2 (TVV2) (AF127178). The RdRp gene of DsRV1 contains all eight conserved motifs (Fig. 4) found in the RdRp gene of most dsRNA viruses (Bruenn 1993). The third methionine (nt. 1500) was



considered to be the likely start codon of ORF2. It is in a more favourable context for translation initiation compared to the first (nt. 1319) and second methionine (nt. 1473) after the stop codon of ORF1, as it has a purine in position -3 and +1 (AAAAUGA) (Kozak 1991). The 219 nucleotides after the stop codon of ORF1 did not have any significant sequence homology to other known viral sequences. Furthermore, DsRV1 has a 5' UTR (untranslated region) of 29 bases and a 3' UTR of 186 bases.

### **Amplification of genomic DNA**

Despite using various reaction conditions, there was no amplification from the genomic DNA using ORF1- and ORF2-specific primers (Fig. 2a). Amplification was obtained from the genomic DNA using ITS1 and ITS4 primers as positive control, as well as from the diluted RT-PCR products using the same primers as initially used to amplify the dsRNA.

### **Phylogenetic relationships**

A most parsimonious cladogram was generated from the amino acid alignments of the RdRps from DsRV1 and 29 other viruses belonging to the *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* and *Endornavirus* (Fig. 5). DsRV1 grouped with *Phlebiopsis gigantea* mycovirus dsRNA element 2 (PgV2), closest to *Helminthosporium victoriae* 145S virus (Hv145SV), *Penicillium chrysogenum* virus (PcV) and *Phlebiopsis gigantea* mycovirus dsRNA element 1 (PgV1). Hv145SV and PcV belong to the *Chrysoviridae*, while PgV1 and PgV2 have not yet been classified. Other than ObrV (*Operophtera brumata* reovirus) and FgV-DK21 (*Fusarium graminearum* virus DK21), all the viruses included in the phylogeny, grouped in two major clades. One of the major clades included DsRV1, PgV2, PgV1, viruses belonging to the *Chrysoviridae*, *Totiviridae*, *Hypoviridae* and those of the genus *Endornavirus*. The other clade included viruses residing in the *Partitiviridae* and the genus *Mycoreovirus*. Viruses belonging to

the three genera residing in the *Totiviridae* i.e. *Totivirus*, *Leishmaniavirus* and *Giardiavirus* grouped accordingly except the *Giardia lamblia virus* (*Giardiavirus*) that was more closely related to viruses in the *Hypoviridae* and the genus *Endornavirus* than to the other two genera (*Totivirus* and *Leishmaniavirus*) in the same family. The mycoreoviruses grouped separately from the insect reovirus, *Operophtera brumata reovirus* included in this study.

## DISCUSSION

The genome of a dsRNA element commonly associated with *D. scrobiculata* was sequenced and characterized in the study and the name *Diplodia scrobiculata* RNA virus 1 (DsRV1) has been proposed for it. DsRV1 is unencapsidated with a monopartite genome. Three smaller dsRNA segments that were isolated together with DsRV1 were shown to be deletion mutants of the virus. Phylogenetically, DsRV1 grouped most closely to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2) (GenBank accession number CAJ34335). Its next closest relatives are viruses belonging to the *Chrysoviridae* (Hv145SV and PcV) (Ghabrial *et al.* 2002; Jiang & Ghabrial 2004).

DsRV1 was isolated from a Californian *D. scrobiculata* isolate and has a genome size of 5018 bp constituting two ORFs. The first ORF codes for a putative polypeptide with relatively high sequence homology to proteins of the class E VPS machinery. The second ORF codes for a RdRp containing all eight conserved motifs found in the RdRp genes of most dsRNA viruses (Bruenn 1993). The method by which DsRV1 translates ORF2 is unknown, as the two ORFs do not overlap to enable translation to occur via ribosomal frameshifting or by internal initiation (Ghabrial 1998). The stretch of untranslated nucleotides between the two ORFs presumably has a structural function in positioning the AUG start codon of ORF2 in a suitable configuration for ribosomal access and translation initiation.

The role of the putative polypeptide encoded by ORF1 of DsRV1 could be to assist in the formation of sub-cellular compartments to protect this unencapsulated virus. Alternatively, it could play a role in virus transmission. Proteins of the VPS machinery are associated with mammalian and yeast cells and have also been reported from fungi where they sort endosomal membrane proteins to multivesicular bodies (MVB) for transport to the lysosomes where they are degraded (Reggiori & Pelham 2001; Iwaki *et al.* 2007). In retroviruses, rhabdoviruses and filoviruses, these proteins have been reported to interact with specific domains (L- or late domains) in the viral GAG-proteins to mediate viral budding or to act as adapters, linking viral L domains with the cellular VPS machinery for efficient viral particle release (Harty *et al.* 2000; Martin-Serrano *et al.* 2003). No mycoviruses have previously been reported to encode for an equivalent polypeptide.

DsRV1 probably obtained a VSP-like protein from its host and it is evolving more rapidly than its cellular homolog. This is consistent with the fact that viruses can obtain genes from their hosts (Khatchikian *et al.* 1989; McGeoch 2001) and it is known that cellular proteins sometimes assist in viral replication and transcription (Lai 1998). Host gene capture is more common in DNA viruses where it represents a mechanism to evade host immune responses (Domingo *et al.* 1998). Host gene capture has, however been reported from RNA viruses for example the ubiquitin-coding gene reported from a togavirus (Meyers *et al.* 1989) and the putative UDP glycosyltransferase gene from *Phytophthora* endornavirus (PEV1) (Hacker *et al.* 2005). In the totivirus, *Helminthosporium victoriae* 190S virus (Hv190sV), a cellular protein with sequence similarity to alcohol oxidases of methylotrophic yeasts was also found to co-purify with viral dsRNA (Soldevila *et al.* 2000; Soldevila & Ghabrial 2001).

We hypothesize that DsRV1, like viruses belonging to the *Hypoviridae* and the genus *Endornavirus*, is associated with cytoplasmic vesicles as it does not have rigid symmetrical structures encoded by inner and outer capsid proteins. Hypoviruses are enveloped in pleomorphic vesicles surrounded by rough endoplasmatic reticulum (Nuss *et al.* 2005). Viruses in the genus *Endornavirus* have unencapsidated dsRNA genomes associated with RdRp activity in cytoplasmic vesicles (Gibbs *et al.* 2005). These structural features of dsRNA's associated with vesicles are characteristic of a replicative intermediate of a ssRNA virus (Jacob-Wilk *et al.* 2006). DsRV1 and other unencapsulated dsRNA viruses therefore, probably had a ssRNA progenitor.

Based on the RdRp (ORF2), DsRV1 is phylogenetically most closely related to PgV2 (GenBank accession number CAJ34335), a dsRNA element isolated from *Phlebiopsis gigantea* that has not yet been assigned family status. ORF1 of both DsRV1 and PgV2, furthermore, encodes hypothetical proteins with no significant homology. The closest relatives to DsRV1 and PgV2 are another dsRNA element from *P. gigantea* (PgV1) and viruses belonging to the *Chrysoviridae* (Hv145SV and PcV) (Ghabrial *et al.* 2002; Jiang & Ghabrial 2004). The *Chrysoviridae* represents a family newly erected to accommodate mycoviruses with multipartite dsRNA genomes of three to four segments (Ghabrial & Castón 2005), previously considered to be part of the genus *Chrysovirus* in the *Partitiviridae* (Jiang & Ghabrial 2004). DsRV1 does have four segments but only one was shown to be functional. Based on the RdRp phylogeny and the unique genome organization of DsRV1, it appears that this virus and its relative (PgV2) occurring in *P. gigantea*, represents a new virus family.

DsRV1 shares little sequence homology with SsRV1 and SsRV2 that occur in the ascomycete fungus *D. pinea*, which is closely related to the host of DsRV1. DsRV1 is in fact more closely

related to dsRNA elements from a basidiomycete. Preisig *et al.* (1998) also reported limited sequence homology between SsRV1 and SsRV2. The existence of three unrelated viruses in two closely related fungal species suggest that they have polyphyletic and separate origins. In a recent study, De Wet *et al.* (2008) showed that DsRV1 always occurs in combination with SsRV1 and/or SsRV2.

DsRV1 is mainly found in association with *D. scrobiculata* populations that have been reported to have high allelic diversities, a history of recombination and/or mutation and potentially the existence of a cryptic sexual cycle (Burgess *et al.* 2004a). SsRV1 and SsRV2, on the other hand are mainly found in association with *D. pinea* populations that have low genetic diversities and a history of asexual recombination (Burgess *et al.* 2004b). As mycoviruses are believed to co-evolve and co-adapt with their fungal hosts (Ghabrial 1998), the genetic variation in DsRV1 could thus be the result of mutation and recombination together with its constantly evolving host (*D. scrobiculata*) to ensure adaptability to changing environments.

The ecological role of DsRV1 is unknown. In the case of SsRV1 and SsRV2, it has been shown that reduced virulence or slower growth in *D. pinea* could not be linked to the presence of these dsRNA elements (Steenkamp *et al.* 1998; De Wet *et al.* 2001). DsRV1, SsRV1 and SsRV2 occur in various combinations in their two related fungal hosts, *D. pinea* and *D. scrobiculata* without any clear pattern of association. The manner in which they interact with each other and their possible role in the biology of their pine pathogen hosts will form the basis of future studies.

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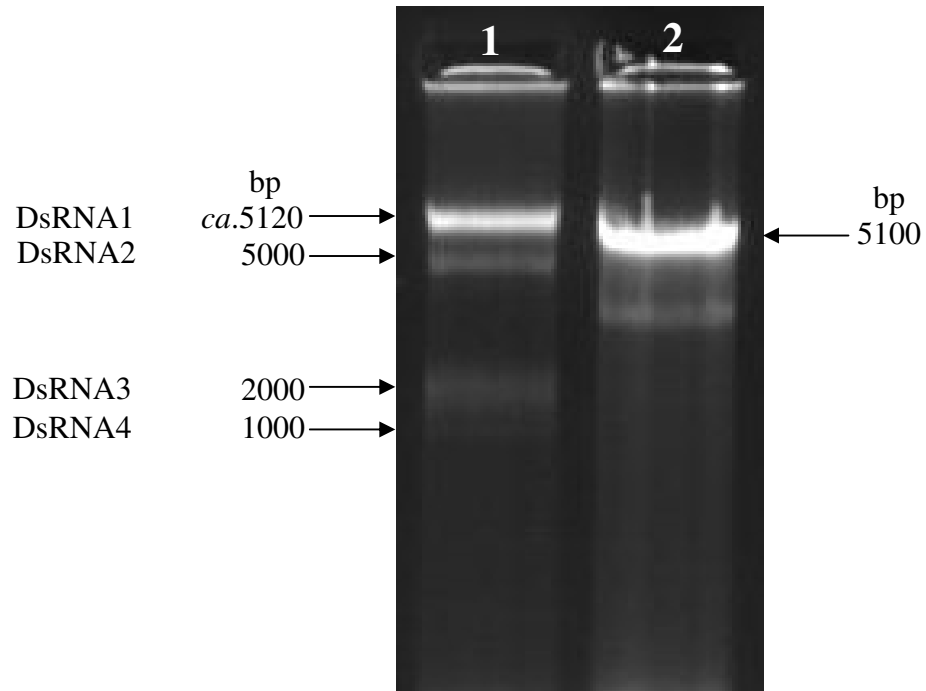
**Table 1.** Names, acronyms and accession numbers of all viruses included in the phylogenetic comparison.

Virus name	Acronym	Accession number	*Family/Genus
<i>Diplodia scrobiculata</i> RNA virus 1	DsRV1	EU547739	Unknown
<i>Sphaeropsis sapinea</i> RNA virus 1	SsRV1	NP047558	<i>Totiviridae</i>
<i>Sphaeropsis sapinea</i> RNA virus 2	SsRV2	NP047560	"
<i>Gremmeniella abietina</i> RNA virus L1	GaV-L1	NP624332	"
<i>Gremmeniella abietina</i> RNA virus L2	GaV-L2	YP044807	"
<i>Giardia lamblia</i> virus	GLV	NP620070	"
<i>Helminthosporium victoriae</i> 190S virus	Hv190sV	NP619670	"
<i>Leishmania</i> RNA virus 1-1	LRV1-1	NP043465	"
<i>Leishmania</i> RNA virus 2-1	LRV2-1	NP041191	"
<i>Trichomonas vaginalis</i> virus 2	TVV2	AF127178	"
<i>Helicobasidium mompa</i> virus no. 17	HmV17	NP898833	"
<i>Botryotinia fuckeliana</i> totivirus	BfV	CAM33265	"
<i>Ophiostoma minus</i> virus	OmV	CAJ34336	"
<i>Phlebiopsis gigantea</i> dsRNA 1	PgV1	CAJ34333	Unknown
<i>Phlebiopsis gigantea</i> dsRNA 2	PgV2	CAJ34335	"
<i>Ophiostoma partitivirus</i> 1	OPV1	CAJ31886	<i>Partitiviridae</i>
<i>Ophiostoma quercus</i> partitivirus	OqPV	CAJ34337	"
<i>Aspergillus ochraceus</i> virus	AoV	ABV30675	"
Black raspberry cryptic virus	BRCV	ABU55400	"
<i>Helicobasidium mompa</i> mycovirus	HmMV	BAC23065	"
<i>Vicia</i> cryptic virus	VCV	ABN71234	"
<i>Penicillium chrysogenum</i> virus	PcV	YP392482	<i>Chrysoviridae</i>
<i>Helminthosporium victoriae</i> 145S virus	Hv145sV	YP052858	"
<i>Cryphonectria hypovirus</i> 1	CHV1	NP041091	<i>Hypoviridae</i>
<i>Cryphonectria hypovirus</i> 1-EP713	CHV1-EP	O04350	"
<i>Operophtera brumata</i> reovirus	ObRV	ABB17205	<i>Reoviridae</i>
Mycoreovirus-1/ <i>Cryphonectria parasitica</i> 9B21	MYRV1/Cp9B21	BAD51414	"
Mycoreovirus-3/ <i>Rosellinia necatrix</i> W370	MYRV3/RnW370	YP392478	"
<i>Helicobasidium mompa</i> endornavirus	HmEV	BAE94538	<i>Endornavirus</i>
<i>Phytophthora</i> endornavirus	PEV	YP241110	"
<i>Fusarium graminearum</i> virus-DK21	FgV-DK21	YP223920	Unknown
<i>Cucurbit yellows-associated virus</i>	CYV	CAA63099	<i>Potyviridae</i>

\* Unknown refers to viruses not assigned to a specific family or genus.



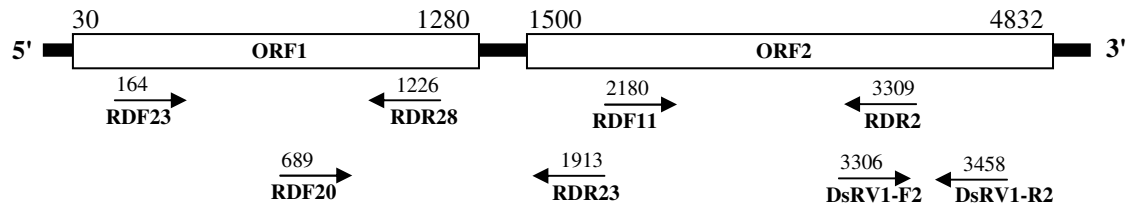
**Figure 1.** A 1 % agarose gel showing the dsRNA segments isolated from *D. scrobiculata* (Lane 1) compared to SsRV1 and SsRV2 isolated from *D. pinea* (Lane 2).



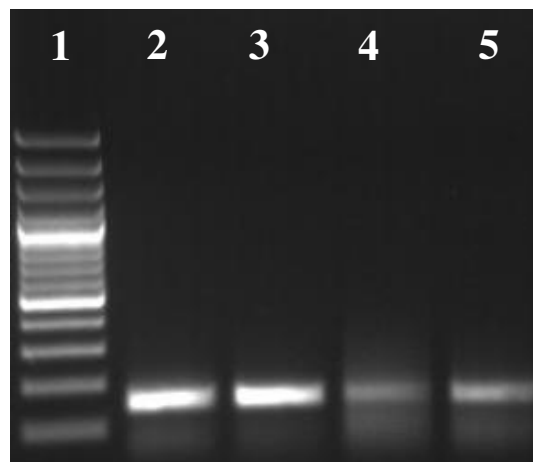
**Figure 2.** (a) A schematic representation of the genome organization of DsRV1. The white blocks represent the coding regions and the black blocks the untranslated regions. The ORF1- and ORF2-specific primers are indicated with arrows in the direction they amplify. The position of the primers on the genome is indicated above the arrow and the primer name below the arrow.

(b) A 1 % agarose gel showing RT-PCR products using the primer pair (DsRV1-F2 and DsRV1-R2) on the four dsRNA segments isolated from *D. scrobiculata*. Lane 1 = 100 bp ladder, Lane 2 = dsRNA1, Lane 3 = dsRNA2, Lane 4 = dsRNA3, Lane 5 = dsRNA4.

(a)



(b)





**Figure 3.** Amino acid alignments of the putative gene product encoded by ORF1 of DsRV1 (EU547739) and a protein belonging to the Class E vacuolar protein-sorting (VPS) machinery (Q0U6X7). Dark shading indicates identical amino acids and lighter shading indicates 60 % similar amino acids.



```

      10      20      30      40      50
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
MFRAQSNIFDDVVVKATDENLTSENWEYILDVCDKVGSSDTGAKDAVAAM
-----

      60      70      80      90     100
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
IKRLAHRNANVQLYTLLELANALSQNCGIQMHKELASRSFTDAMLRLANDR
-----

      110     120     130     140     150
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
NTHQAVKAKILERMGEWSEMFSDPDLGIMEGAYMKLKTQ-----
-----MGEWTEMFASNFDLGIMEQAYMRLKTQSACDQLWFGV
-----

      160     170     180     190     200
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
-----NPNLRAPSPKPKQTQISDSDRQKEEELQMALAMSIKESKCATP
STDQRVVDPNLRPPSPKPKQTQITDSDRQKEEELQMALALSVKES-GTEP
-----

      210     220     230     240     250
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
SAAKANAPQESNAGSSSQAAPAPQPVQPQTAAATVSRVRLFDQPSSEPG
SAARSNPQAKAPQSSAPEQEQHQAIASGTTAATVSRVRLYDFTPSEPG
-----

      260     270     280     290     300
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
ELQFKKGDIIAVLESVYKDWWKGLRGNIGIFPLNYVEKLQDPTREELEK
ELAFRKGDI IAVLESVYKDWWKGLRGGTIGIFPLNYVEKLQDPTKEELER
-----

      310     320     330     340     350
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EAQTFAEVFAQIRNVEKLLALLSTNTQAGGGDGRDNEEITELYHSTLAIK
EAQMEAEVFAEIKNVEKLLALLSTSSA---DARDNEEITSLYHKIVSIR
-----

      360     370     380     390     400
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PKLIELIGKYSQKDDFTQINEKFIKARDYESTLEASMSQPPQPSYGSR
PKLIELIGKYSQKKGKWNLLRFVFCWPLIVFQMTSRSSRTRSSRHAATK
-----

      410     420     430     440     450
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PPYGYNAPPSPSNYTGYPSSPPQYGYGAGAPQGSAPQYPPVGANPAF
P----SLKFRRCNRQSPATVVNP-----MATAP----APRRLRLRTVAI
-----

      460     470     480     490     500
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
FMVPPAGEQRPOQTPOPGPPSDPYSLPQGRVPIGGRPQSYAPQELATAH
LLKDLLRN--SFTRLLOPQTKATDILLRMGLSPS----RWLSPHKHLLLL
-----

      510     520     530     540     550
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
YDSPVDNRHSFAGFSQPQGAPSAPOGYEYPPSQAPPYPPQOGAPLQGP
RWLVLDQRMASSTSRSACLSVEGPHWP-----FPRKWRP
-----

      560     570     580     590     600
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PGQONPYEQISSPPTHQPPSDPYSQPPPQVGHGYPPQPAHAPPAPPGA
TAQWTFGRLRASKIRK-----
-----

      610     620     630
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
SSSPAPAQGYLPYRPPGQAPSAPPVGGGGDEGFYR
-----

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**Figure 4.** Partial amino acid alignments of the RdRp genes for a set of dsRNA viruses, showing the eight conserved motifs (marked A-H). Viruses included were DsRV1 (*Diplodia scrobiculata* RNA virus1) (EU547739), PgV1 (*Phlebiopsis gigantea* mycovirus 1) (CAJ34333), PgV2 (*Phlebiopsis gigantea* mycovirus 2) (CAJ34335), TVV2 (*Trichomonas vaginalis* virus 2) (AF127178), SsRV1 (*Sphaeropsis sapinea* RNA virus 1) (NP047558), SsRV2 (*Sphaeropsis sapinea* RNA virus 2) (NP047560), GaVL1 (*Gremmeniella abietina* RNA virus L1) (NP624332), Hv190SV (*Helminthosporium victoriae* 190S virus) (NP619670), LRV2-1 (*Leishmania* RNA virus 2-1) (NP041191), Hv145SV (*Helminthosporium victoriae* 145S virus) (YP052858), PcV (*Penicillium chrysogenum* virus) (YP392482) and FgV-DK21 (*Fusarium graminearum* virus DK21) (YP223920). All are members of the *Totiviridae* except PcV and Hv145SV belonging to the *Chrysoviridae* and DsRV1, PgV1 and PgV2 that have not been assigned to a virus family. Dark shading indicates identical amino acids and lighter shading indicates 50 % similar amino acids.

	<b>A</b>		<b>B</b>	<b>C</b>				<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>		
	1080	1090	1170	1240	1250	1260	1270	1350	1420	1430	1465	1505	1520	1530
<b>DsRV1</b>	LPGRNE	VFVAVSHVGD	MFMRR	WAASG	KFENG	KRRIT	WNTSLTHYVAQGFLDLVE	DYADFNINHS	SLASGERATSEFVN	TVLSRAY	GDDVF	EFLRQ	GYPFR	SAMGLFSGEY
<b>PgV2</b>	LPGPSE	LHKFD	PQAEILQR	WAASG	KFEKG	KRRAI	WNTAIEHYLFQAYILDIID	DFSDFNINHQ	CLASGERATSE	TNILLSRVY	GDDVF	EFLRL	GYPFR	AGLGLISGEF
<b>PgV1</b>	LVGRAQ	DELLG	FDGYVEKR	LTPSG	KTESG	LRLRQI	IPGEIHQWLIESIAMYRIE	DYADFNYLHT	SLWSGWR	TTTMINNMNLVY	GDDGD	EYLRI	GSIR	SCASFVGGDL
<b>TVV2</b>	LQGRG	VTSSDA	KRDLTHR	WVKKG	KLEHG	KTRFI	YNCDTVSYIYFDYIILNYIE	DYDFNSQHS	TLMSGRR	GTTYISSVLNEVY	GDDVL	EFLRL	GYPAR	AISSLVSGNW
<b>SsRV2</b>	LQGRA	VQPAD	MHSEAIR	WAVNG	KLEHG	KTRAI	FACDTLNYLAFEHLLASVE	DFDDFNSHHS	TLPSGHR	GTTFIVNSVLNAAY	GDDVY	EFLRM	GYLAR	SVASFVSGNW
<b>SsRV1</b>	LLGRA	VSTID	LAHEAEYR	WCVNG	KLEHG	KTRAI	FACDTRSIFYAFEWLLGATQ	DYDDFNSHHS	TLMSGHR	GTTFINSVLNKAY	GDDVY	EFLRN	GYFAR	AVASTVSGNW
<b>GaVL1</b>	LLGRA	AGPAD	LSEEAR	WAVNG	KLEAG	KTRAI	FACDTVNYLAFEHLLAPVE	DYDNFNSQHS	TLMSGHR	ATTFINSVLNAAAY	GDDVY	EFLRL	GYLAR	SVASFVSGNW
<b>Hv190SV</b>	LQGRY	DRTLD	MDHEVESR	WAVNG	KLENG	KDRAI	FACDTRSIFYAFETYWLTPIE	DFEDFNNSQHS	TLMSGHR	ATTFINTILNTAY	GDDVI	EFLRV	GYVAR	AIASCVSGNW
<b>LRV2-1</b>	LRGRG	IAELD	VIAEAKQR	WAANG	KLEHG	KSRL	LACDTLSYLWFEEYYLKPVE	DWANFNVQHS	GLYSGWR	GTTWDNTVLNGCY	GDDVD	EFFRV	ASPVR	GLATFVAGNW
<b>Hv145SV</b>	LLGRR	IFIAE	DAEIKDG	WMTKG	AVEKLN	ENGHKDRVL	LPGGLLHYIVFAYVLRCAE	DWADFNEQHS	GLYSGWR	GTTWINTVLFNFCY	GDDID	EFFRN	ASPTR	ALASFVAGDW
<b>PcV</b>	LVGRG	EYTV	DEMAEVELR	WLTKG	TQIKY	EVGKK	RTL-LPGTLVHFVVETTYVLYLAE	DQSNFDRQPD	GLPSG	WKWIALLGALINVTQ	-DDIA	EFLRR	GYPAR	MMIKLLYQL
<b>GLV</b>	YICSR	GYVD	TGFKALDIYDILSQ	YKNPS	TTGSC	YIGYGR	SFNKWSIYGAYPTEEIYRLALYG							

**Figure 5.** The most parsimonious phylogram generated after a phylogenetic analysis of the amino acid sequences of the RdRp genes of DsRV1 (EU547739) compared to viruses of the *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridae*, *Reoviridae* and the genus *Endornavirus*. Viruses included were SsRV1 (*Sphaeropsis sapinea* RNA virus 1) (NP047558), SsRV2 (*Sphaeropsis sapinea* RNA virus 2) (NP047560), GaV-L1 (*Gremmeniella abietina* RNA virus L1) (NP624332), GaV-L2 (*Gremmeniella abietina* RNA virus L2) (YP044807), GLV (*Giardia lamblia* virus) (NP620070), Hv190SV (*Helminthosporium victoriae* 190S virus) (NP619670), LRV1-1 (*Leishmania* RNA virus 1-1) (NP043465), LRV2-1 (*Leishmania* RNA virus 2-1) (NP041191), TVV2 (*Trichomonas vaginalis* virus 2) (AF127178), HmV17 (*Helicobasidium mompa* virus no. 17) (NP898833), BfV (*Botryotinia fuckeliana* totivirus) (CAM33265), OmV (*Ophiostoma minus* virus) (CAJ34336), *Phlebiopsis gigantea* mycovirus 1 (PgV1) (CAJ34333), *Phlebiopsis gigantea* mycovirus 2 (PgV2) (CAJ34335), OPV1 (*Ophiostoma partitivirus* 1) (CAJ31886), OqPV (*Ophiostoma quercus* partitivirus) (CAJ34337), AoV (*Aspergillus ochraceus* virus) (ABV30675), BRCV (Black raspberry cryptic virus) (ABU55400), HmMV (*Helicobasidium mompa* mycovirus) (BAC23065), VCV (*Vicia cryptic* virus) (ABN71234), PcV (*Penicillium chrysogenum* virus) (YP392482), Hv145sV (*Helminthosporium victoriae* 145S virus) (YP052858), CHV1 (*Cryphonectria hypovirus* 1) (NP041091), CHV1-EP (*Cryphonectria hypovirus* 1-EP713) (Q04350), ObRV (*Operophtera brumata* reovirus) (ABB17205), MYRV1/Cp9B21 (Mycoreovirus-1/*Cryphonectria parasitica* 9B21) (BAD51414), MYRV3/RnW370 (Mycoreovirus-3/*Rosellinia necatrix* W370) (YP392478), HmEV (*Helicobasidium mompa* endornavirus) (BAE94538), PEV1 (*Phytophthora* endornavirus) (YP241110) and FgV-DK21 (*Fusarium graminearum* virus-DK21) (YP223920). The cucurbit yellows-associated virus (CYV) (CAA63099), a (+) ssRNA plant virus was used as outgroup.

