# Characterization of a novel dsRNA element in the pine endophytic fungus, *Diplodia scrobiculata*

[1] Juanita De Wet, [2] Wubetu Bihon, [1] Oliver Preisig, [2] Brenda D. Wingfield and [2] Michael J. Wingfield

[2]Departments of Genetics, [1] Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Corresponding author: wubetu.bihon@fabi.up.ac.za

## 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 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 similarity 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 identity with SsRV1 or SsRV2 and they probably have different recent evolutionary origins.

---

**Keywords**: *Diplodia scrobiculata* RNA virus, SsRV1, SsRV2, dsRNA elements, mycoviruses

## INTRODUCTION

*Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. is an opportunistic pathogen of mainly *Pinus* spp. that co-exists with the well-known pine pathogen, *D. pinea* (Desm.) Kickx (=*Sphaeropsis sapinea*), where their host ranges overlap [8, 37]. This fungus was previously known as the B morphotype of *D. pinea* [11, 37, 45]. 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 [44, 47].

*Diplodia scrobiculata* and *D. pinea* can be distinguished based on morphology, distribution, virulence and DNA sequence comparisons [11, 13, 14, 37, 45]. *Diplodia scrobiculata* has a low level of virulence with restricted distribution in the northern hemisphere except the recent report in South Africa [2]. However, *D. pinea* can be highly virulent and has a world-wide distribution [4, 7, 8, 9]. Phylogenetic analysis using DNA sequence of protein coding genes and microsatellite markers differentiated the two species [14]. The genetic and genotypic diversity of *D. scrobiculata* and *D. pinea* populations are high especially in the introduced environment. These suggest a recent history of recombination and/or mutation [3, 8].

Several dsRNA elements of different sizes have been reported from both *D. pinea* and *D. scrobiculata* [1, 12, 38, 43, 48]. 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) [38]. They are characterized by monopartite dsRNA genomes, 5 kb size, with two ORFs. One of these ORFs codes for a capsid polypeptide (CP) and the other for a RNA dependent RNA polymerase (RdRp). Based on these characteristics and phylogenetic relationships, they have been shown to be closely related to viruses in the genus *Totivirus* and the family *Totiviridae* [38]. In a recent study, a third dsRNA element was isolated from a Californian *D. scrobiculata* isolate [15] which is considered in this study.

Multiple infections with different viruses are common in fungi [6]. The frequency and distribution of the three viruses associated with *D. pinea* and *D. scrobiculata* was determined using Real-time PCR and virus-specific primers [15]. 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. 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 [6, 17]. 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 [12, 43]. However, in a study conducted by Adams et al. [1], 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 comparison 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, 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) 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 ºC 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 ºC, washed with 70 % ethanol, dried and re-suspended in 50 µl DEPC-treated $dH_2O$. The isolated dsRNA was separated on a 1 % agarose (w/v) gel (Promega) 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 ºC and stored at -20 ºC 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, Germany). The dsRNA and random hexamer primers were denatured for 10 min at 99 ºC 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-proof reader cloning kit (Lucigen® Corporation, Middleton, WI, USA). PCR amplified inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Germany) 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, Germany). The 50 µl reaction mixture containing 1x RT-PCR buffer (1.5 mM

$MgCl_2$ and DMSO), 5 mM DTT, 0.2 mM each dNTP, 5 U RNase Inhibitor, 1 µl enzyme mix, 0.4 uM each primer and the dsRNA template. The primers and dsRNA were firstly denatured for 10 min at 99 ºC 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 ºC. This was followed by PCR amplification of 1 cycle at 94 ºC, 10 cycles of 94 ºC for 30 s, 50 ºC for 30 s and 68 ºC for 2 min, 25 cycles of 94 ºC for 30 s, 50 ºC for 30 s and 68 ºC for 2 min with a cycle elongation of 5 s per cycle and finally an elongation step of 10 min at 68 ºC.

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, Germany) 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) and IPTG (Fermentas Life Sciences). Colony PCR, 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) [30, 34] and RLM-RACE (RNA ligase-mediated amplification of cDNA ends) [10] 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: 5'-GCATTCGACCCGGGTT-3') to the dsRNA using T4 RNA ligase (Roche Diagnostics, Germany). 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: 5'-AACCCGGGTCGTATGC-3') complementary to PC4 with the Fermentas First strand cDNA synthesis kit (Fermentas Life Sciences). 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 the Retsch MM301 homogenizer (30 freq/s; 30 s), followed by the extraction of DNA using the technique described by Raeder & Broda [39] DNA was stored at -20 ºC 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'-CCGCCATTTCCTGGGGAAAGGCC-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 [46] 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 $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgCl_2$, 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 Biochemicals). The following temperature profile was used: 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® BigDye® Terminator v3.1 Cycle Sequencing kit and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster City, CA 94404 USA). Reactions were done using protocols recommended by the manufacturers. Sequence data were processed using Chromas version 2.3 (Technelysium) and contigs assembled using Sequencher 4.1.4 (Gene Codes Corporation). Alignments of overlapping contigs were done in BioEdit Sequence Alignment Editor (Tom Hall Isis Pharmaceuticals).

**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 [26]. 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 [36].

## 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 ºC 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), suggest that the three smaller dsRNA bands might either represent fragments of the larger RNA molecule or deletion mutants. Additional research will be needed to unambiguously identify the smaller dsRNAs (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 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 (CGU**AUG**G). 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 of

*Phlebiopsis gigantea* mycovirus 2 (PgV2), 25 % identity and 36 % similarity to the RdRp of *Sphaeropsis sapinea* RNA virus 1 (SsRV1) and 24 % identity and 38 % similarity to the RdRp of *Trichomonas vaginalis* virus 2 (TVV2). The RdRp of DsRV1 contains all eight conserved motifs (Fig. 4) found in the RdRp of most dsRNA viruses [5]. The third methionine (nt. 1500) was considered to be 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 (A**AUG**A) [28]. The 219 nucleotides after the stop codon of ORF1 did not have any significant sequence homology to other known viral sequences. DsRV1 furthermore, 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, no amplification was obtained 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 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. All the viruses included in the phylogeny, grouped in two major clades except for *Operophtera brumata* reovirus (ObRV) and *Fusarium graminearum* virus DK21 (FgV-DK21). One of the major clades included DsRV1, PgV2, PgV1, viruses of the *Chrysoviridae*, *Totiviridae*, *Hypoviridae* and those of the genus *Endornavirus*. The other clade included viruses of the *Partitiviridae* and the genus *Mycoreovirus*. Viruses belonging to the three genera of 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 may be defective RNAs or deletion mutants or degraded from the largest fragments. Phylogenetically, DsRV1 grouped most closely to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2). Its next closest relatives are viruses belonging to the *Chrysoviridae* (Hv145sV and PcV) [18, 25].

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 similarity 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 [5]. 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 [17]. The stretch of untranslated nucleotides between the two ORFs probably presumably has a structural function in positioning the AUG starting codon of ORF2 in a suitable configuration for ribosomal access and translation initiation.

The role of the putative polypeptide encoded by ORF1 of DsRV1 is could be to assist in the formation of sub-cellular compartments to protect this unencapsulated virus. Alternatively this polypeptide 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 multivesical bodies (MVB) for transport to the lysosomes to be degraded [23, 40]. 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 [22, 31]. 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 [27, 32] and it is known that cellular proteins sometimes assist in viral replication and transcription [29]. Host gene capture is more common in DNA viruses where it represents a

mechanism to evade host immune responses [16]. Host gene capture has, however been reported from RNA viruses for example the ubiquitin-coding gene reported from a togavirus [33] and the putative UDP glycosyltransferanse gene from *Phytophthora* endornavirus (PEV1) [21]. 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 [41, 42].

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 [35]. Viruses in the genus *Endornavirus* have unencapsidated dsRNA genomes associated with RdRp activity in cytoplasmic vesicles [20]. These structural features of dsRNA's associated with vesicles are characteristic of a replicative intermediate of a ssRNA virus [24]. DsRV1 and other unencapsulated mycoviruses 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 elemet isolated from *Phlebiopsis gigantea* that has not yet been assigned family status. The closest relatives to DsRV1 and PgV2 are another dsRNA element from *P. gigantea* (PgV1) and viruses belonging to the *Chrysoviridae* (Hv145SV and PcV) [18, 25]. The *Chrysoviridae* represents a family newly erected to accommodate mycoviruses with multipartite dsRNA genomes of three to four segments [19], previously considered to be part of the genus *Chrysovirus* in the *Partitiviridae* [25]. 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 similarity with SsRV1 and SsRV2 that occur in the ascomycete *D. pinea*, which is closely related to the host of DsRV1. DsRV1 is in fact more closely related to dsRNA elements from basidiomycetes. Preisig et al. [38] also reported limited sequence homology between SsRV1 and SsRV2. The existence of three unrelated viruses in two closely related fungal species suggest that they are polyphyletic and thus likely have distinct origins. In a recent study, De Wet *et al*. [15] 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 [9]. 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 [8]. As mycoviruses are believed to co-evolve and co-adapt with their fungal hosts [17], genetic variability could be expected in DsRV1 because 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 [12, 43]. 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.

**ACKNOWLEDGEMENTS**

We thank the National Research Foundation (NRF), members of the Tree Pathology Co-operative Programme (TPCP) and the THRIP initiative of the Department of Trade and Industry (DTI), South Africa for financial support.

**REFERENCES**

1. Adams GC, Wu N-T, Eisenberg BE. Virulence and double-stranded RNA in *Sphaeropsis sapinea*. Forest Pathology 2002;32:309-329.
2. Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD. *Diplodia scrobiculata* found in the southern hemisphere. For. Path. 2010a; doi: 10.1111/j.1439-0329.2010.00649.x.
3. Bihon W, Burgess T, Slippers B, Wingfield MJ, Wingfield BD. Distribution of *Diplodia pinea* and its genotypic diversity within asymptomatic *Pinus patula* trees. Australasian Plant Path. (in press); 2010b.
4. Blodgett JT, Stanosz GR. *Sphaeropsis sapinea* morphotypes differ in aggressiveness, but both infect nonwounded red and jack pine. Plant Disease, 1997;81:143-147.
5. Bruenn JA. A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. Nucleic Acids Research. 1993;21:5667-5669.

6. Buck KW. Fungal virology – an overview, in: Buck KW. (Ed), Fungal Virology. 1986; CRC Press: Boca Raton, Florida.
7. Burgess T, Wingfield MJ. Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the Southern Hemisphere. International Forestry Review. 2002;4:56-65.
8. Burgess TI, Wingfield MJ, Wingfield BD. Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology 2004a;33:513-519.
9. Burgess TI, Gordon TR, Wingfield MJ, Wingfield BD. Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. Mycological Research. 2004b;108:1399-1406.
10. Coutts RHA, Livieratos IC. A rapid method for sequencing the 5'- and 3'termini of double-stranded RNA viral templates using RLM-RACE. Journal of Phytopathology. 2003;151:525-527.
11. De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD. Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. Plant Disease. 2000;84:151-156.
12. De Wet J, Wingfield MJ, Preisig O, Wingfield BD. Occurrence of dsRNA elements in isolates of the pine pathogen, *Sphaeropsis sapinea* from diverse geographical regions. Phytopathology. 2001;91:S22.
13. De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD. Characterisation of the 'C' morphotype of the pine pathogen S*phaeropsis sapinea*. Forest Ecology and Management. 2002;161:181-188.

14. De Wet J, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ. Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research. 2003;107: 557-566.

15. De Wet J, Preisig O, Wingfield BD, Wingfield MJ. Patterns of multiple virus infections in the conifer pathogenic fungi, *Diplodia pinea* and *Diplodia scrobiculata*. Journal of Phytopathology. 2008;156:725-731.

16. Domingo E, Baranowski E, Ruiz-Jarabo CM, Martín-Hernández AM, Sáiz JC, Escarmís C. Quasispecies structure and persistence of RNA viruses. Emerging Infectious Diseases. 1998;4:521-527.

17. Ghabrial SA. Origin, adaptation and evolutionary pathways of fungal viruses. Virus genes. 1998;16:119-131.

18. Ghabrial SA, Soldevila AI, Havens WM. Molecular genetics of viruses infecting the plant pathogenic fungus *Helminthosporium vict*oriae, in: Tavantzis SM (Eds), *Molecular Biology of Double-stranded RNA: Concepts and Applications in Agriculture, Forestry and Medicine*, 2002; Chapter 9. CRC Press, Boca Raton, pp. 213-236.

19. Ghabrial SA, Castón JR. *Chrysoviridae*, in: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL. (Eds), *Virus taxonomy*. *Eight Report of the International Committee on taxonomy of viruses*. Elsevier/Academic Press, London; 2005.

20. Gibbs MJ, Pfeiffer P, Fukuhara T. *Endornavirus*, in: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL. (Eds), *Virus taxonomy*. *Eight Report of the International Committee on taxonomy of viruses*. Elsevier/Academic Press, London; 2005.

21. Hacker CV, Brasier CM, Buck KW. A double-stranded RNA from a *Phytophthora* species is related to the plant endornaviruses and contains a putative UDP glycosyltransferase gene. Journal of General Virology. 2005; 86: 1561-1570.

22. Harty RN, Brown ME, Wang G, Huibregtse J, Hayes FP. A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: Implications for filovirus budding. Proceedings of the National Academy of Sciences USA. 2000;97:13871-13876.

23. Iwaki T, Onishi M, Ikeuchi M, Kita A, Sugiura R, Giga-hama Y, Fukui Y, Takegawa K. Essential roles of class E Vps proteins for sorting into multivesicular bodies in *Schizosaccharomyces pombe*. Microbiology. 2007;153:2753-2764.

24. Jacob-Wilk D, Turina M, Van Alfen NK. Mycovirus Cryphonectria Hypovirus 1 elements cofractionate with *trans*-Golgi Network membranes of the fungal host *Cryphonectria parasitica*. Journal of Virology. 2006;80:6588-6596.

25. Jiang D, Ghabrial SA. Molecular characterization of *Penicillium chrysogenum virus*: reconsideration of the taxonomy of the genus *Chrysovirus*. Journal of General Virology. 2004;85:2111-2121.

26. Katoh K, Kuma K-I, Toh H, Miyata T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research. 2005;33:511-518.

27. Khatchikian D, Orlich M, Rott R. Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of a influenza virus. Nature. 1989;340:156-157.

28. Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. Journal of Biological Chemistry. 1991;266:19867-19870.

29. Lai MMC. Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. Virology. 1998;244:1-12.

30. Liu Y-G, Whittier RF. Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics. 1995;25:674-681.

31. Martin-Serrano J, Yaravoy A, Perez-Caballero D, Bieniasz PD. Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. Proceedings of the National Academy of Science USA. 2003;100:12414-12419.

32. McGeoch DJ. Molecular evolution of the γ–Herpesvirinae. Philosophical Transactions of the Royal Society of London, Series B. 2001;356:421-435.

33. Meyers G, Rümenapf T, Thiel H-J. Ubiquitin in a togavirus. Nature. 1989; 341, 491.

34. Nakayama T, Soma M, Rahmutula D, Ozawa Y, Kanmatsuse K. Isolation of the 5'-flanking region of genes by thermal asymmetric interlaced polymerase chain reaction. Medical Science Monitor. 2001;7:345-349.

35. Nuss DL, Hillman BI, Rigling D, Suzuki N *Hypoviridae*, in: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL. (Eds), *Virus taxonomy*. Eight Report of the International Committee on taxonomy of viruses. Elsevier/Academic Press, London. 2005.

36. Page RD. TreeView: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences. 1996; 12: 357-358.

37. Palmer MA, Stewart EL, Wingfield MJ. Variation among isolates of *Sphaeropsis sapinea* in the North Central United States. Phytopathology. 1987;77:944-948.

38. Preisig O, Wingfield BD, Wingfield MJ. Co-infection of a fungal pathogen by two distinct double-stranded RNA viruses. Virology. 1998;252:399-406.

39. Raeder U, Broda P. Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology. 1985;1:17-20.

40. Reggiori F, Pelham HRB. Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. EMBO Journal. 2001;20:5176-5186.

41. Soldevila AI, Havens WM, Ghabrial SA. A cellular protein with an RNA-binding activity co-purifies with viral dsRNA from mycovirus-infected *Helminthosporium victoriae*. Virology. 2000;272:183-190.

42. Soldevila, AI, Ghabrial, SA. A novel alcohol oxidase/RNA binding protein with affinity for mycovirus double-stranded RNA from the filamentous fungus *Hellminthosporium* (*Cochliobolus*) *victoriae*. Journal of Biological Chemistry. 2001;276:4652-4661.

43. Steenkamp ET, Wingfield BD, Swart WJ, Wingfield MJ. Double-stranded RNA and associated virulence in South African isolates of *Sphaeropsis sapinea*. Canadian Journal of Botany. 1998;76:1-7.

44. Swart WJ, Wingfield MJ. Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease. 1991;75:761-766.

45. Wang C-G, Blanchette RA, Jackson WA, Palmer MA. Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. Plant Disease. 1985;69:838-841.

46. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal rRNA genes for phylogenetics, in: Innis MA, Gelfand DH, Sninsky JJ, White TJ. (Eds), *PCR*

*protocols: a guide to methods and applications*. Academic Press, San Diego, USA, 1990; pp. 315-322.

47. Wingfield MJ, Knox-Davies PS. Association of *Diplodia pinea* with a root disease of pines in South Africa. Plant Disease. 1980;64:221-223.
48. Wu N-T, Palmer M, Adams G. The relationship of dsRNA to virulence and morphology in type A and type B *Sphaeropsis sapinea*. Phytopathology. 1989;79:1143.

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

| Virus name | Acronym | Accession number | Reference sequences | Family/Genus |
|---|---|---|---|---|
| *Aspergillus ochraceous virus* | AoV | ABV30675 | - | *Partitiviridae* |
| Black raspberry cryptic virus | BRCV | ABU55400 | - | " |
| *Botryotinia fuckeliana* totivirus | BfV | CAM33265 | - | *Totiviridae* |
| *Cryphonectria hypovirus 1* | CHV1 | - | NP_041091 | *Hypoviridae* |
| *Cryphonectria hypovirus 1-EP713* | CHV1-EP | Q04350 | - | " |
| *Cucurbit yellows-associated virus* | CYV | CAA63099 | - | *Potyviridae* |
| *Diplodia scrobiculata* RNA virus 1 | DsRV1 | EU547739 | - | Unassigned |
| *Fusarium graminearum* virus-DK21 | FgV-DK21 | YP223920 | - | " |
| *Giardia lamblia virus* | GLV | - | NP_620070 | *Totiviridae* |
| *Gremmeniella abietina* RNA virus L1 | GaV-L1 | - | NP_624332 | " |
| *Gremmeniella abietina* RNA virus L2 | GaV-L2 | YP044807 | - | " |
| *Helicobasidium mompa* endornavirus | HmEV | BAE94538 | - | *Endornavirus* |
| *Helicobasidium mompa* mycovirus | HmMV | BAC23065 | - | *Partitiviridae* |
| *Helicobasidium mompa* virus no. 17 | HmV17 | - | NP_898833 | *Totiviridae* |
| *Helminthosporium victoriae 145S virus* | Hv145sV | YP052858 | - | *Chrysoviridae* |
| *Helminthosporium victoriae 190S virus* | Hv190sV | - | NP_619670 | *Totiviridae* |
| *Leishmania RNA virus 1-1* | LRV1-1 | - | NP_043465 | " |
| *Leishmania RNA virus 2-1* | LRV2-1 | - | NP_041191 | " |
| Mycoreovirus-1/*Cryphonectria parasitica* 9B21 | MYRV1/Cp9B21 | BAD51414 | - | *Reoviridae* |
| Mycoreovirus-3/*Rosellinia necatrix* W370 | MYRV3/RnW370 | YP392478 | - | " |
| *Operophtera brumata* reovirus | ObRV | ABB17205 | - | " |
| *Ophiostoma minus* virus | OmV | CAJ34336 | - | *Totiviridae* |
| *Ophiostoma* partitivirus 1 | OPV1 | CAJ31886 | - | *Partitiviridae* |
| *Ophiostoma quercus* partitivirus | OqPV | CAJ34337 | - | " |
| *Penicillium chrysogenum virus* | PcV | YP392482 | - | *Chrysoviridae* |
| *Phlebiopsis gigantea* dsRNA 1 | PgV1 | CAJ34333 | - | Unassigned |
| *Phlebiopsis gigantea* dsRNA 2 | PgV2 | CAJ34335 | - | " |
| *Phytophthora* endornavirus | PEV | YP241110 | - | *Endornavirus* |
| *Sphaeropsis sapinea* RNA virus 1 | SsRV1 | - | NP_047558 | *Totiviridae* |
| *Sphaeropsis sapinea* RNA virus 2 | SsRV2 | - | NP_047560 | " |
| *Trichomonas vaginalis* virus 2 | TVV2 | AF127178 | - | " |
| *Vicia cryptic virus* | VCV | ABN71234 | - | *Partitiviridae* |

**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).

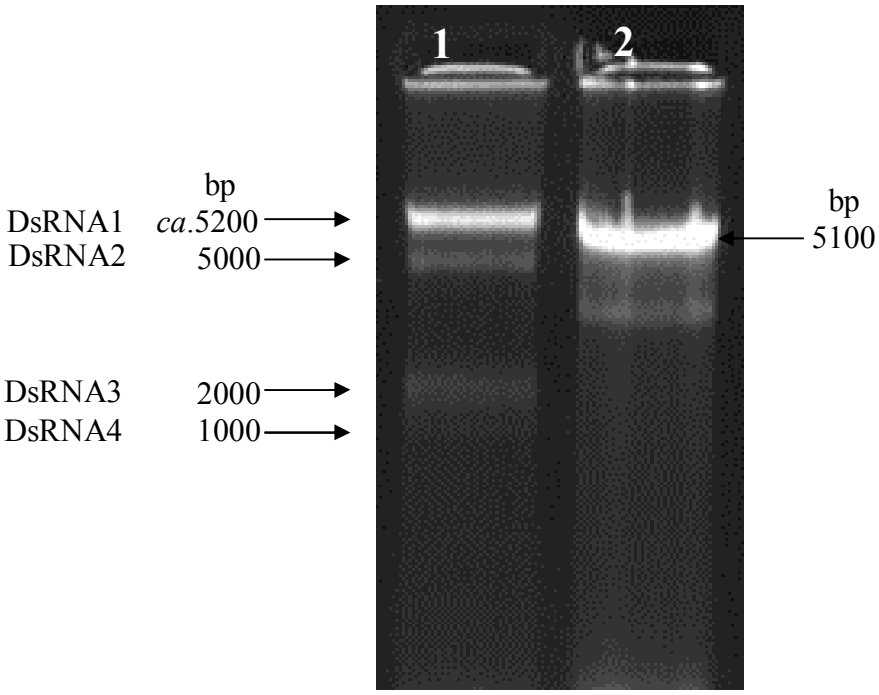
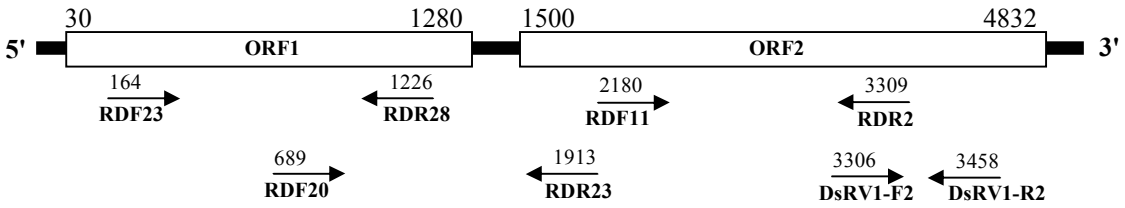


**Figure 2.** (a) A schematic representation of the genome organization of DsRV1. The white blocks represent the coding regions and the black blocks the untranslated regions. ORF1- and ORF2- specific primers are indicated with arrows in the direction they amplify. The position of the primers on the genome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

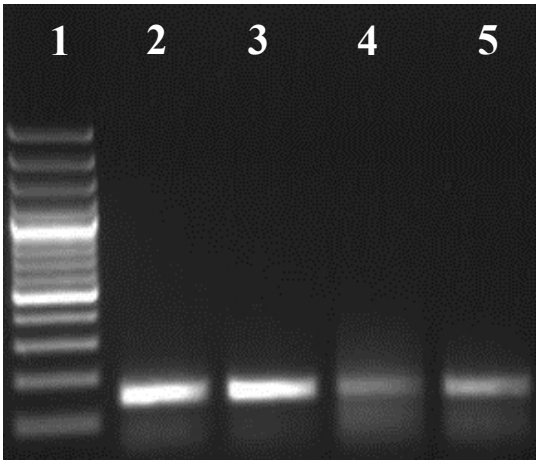


**Figure 3.** Amino acid alignments of the complete ORF1 of DsRV1 (EU547739) and a protein belonging to the Class E vacuolar protein-sorting (VPS) machinery (Q0U6X7). Dark shading indicates identical amino acids and lighter shading indicates 60 % amino acid similarity.

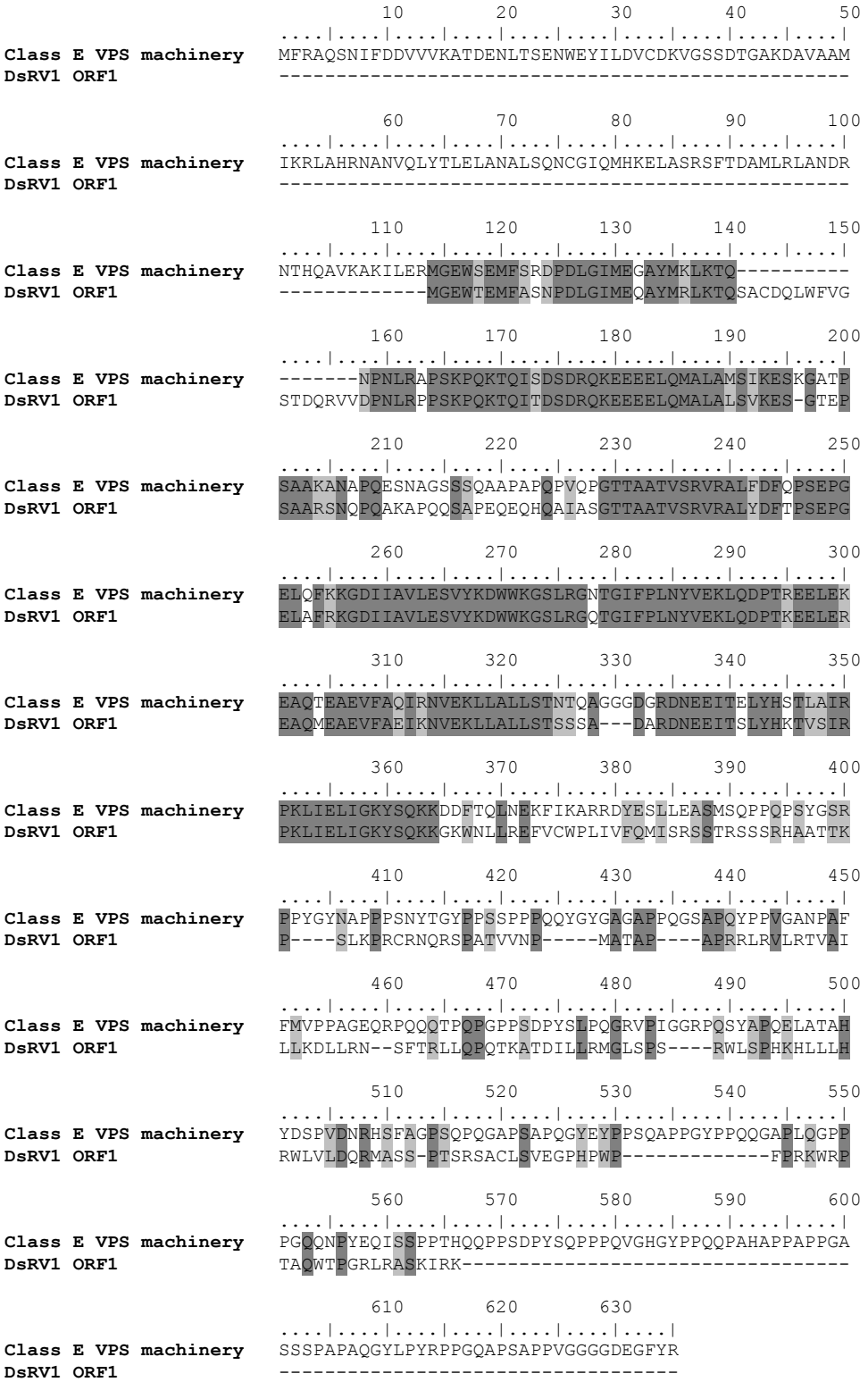


**Figure 4.** Partial amino acid alignments of the RdRp genes for a set of dsRNA viruses, showing the eight conserved motifs (marked A-H). Viruses include here are members or proposed members of the family *Totiviridae* except PcV and Hv145SV belonging to the *Chrysoviridae* and DsRV1, PgV1 and PgV2 that have not been assigned to a virus family. Dark shading indicates identical amino acids and lighter shading indicates 50 % similar amino acids.

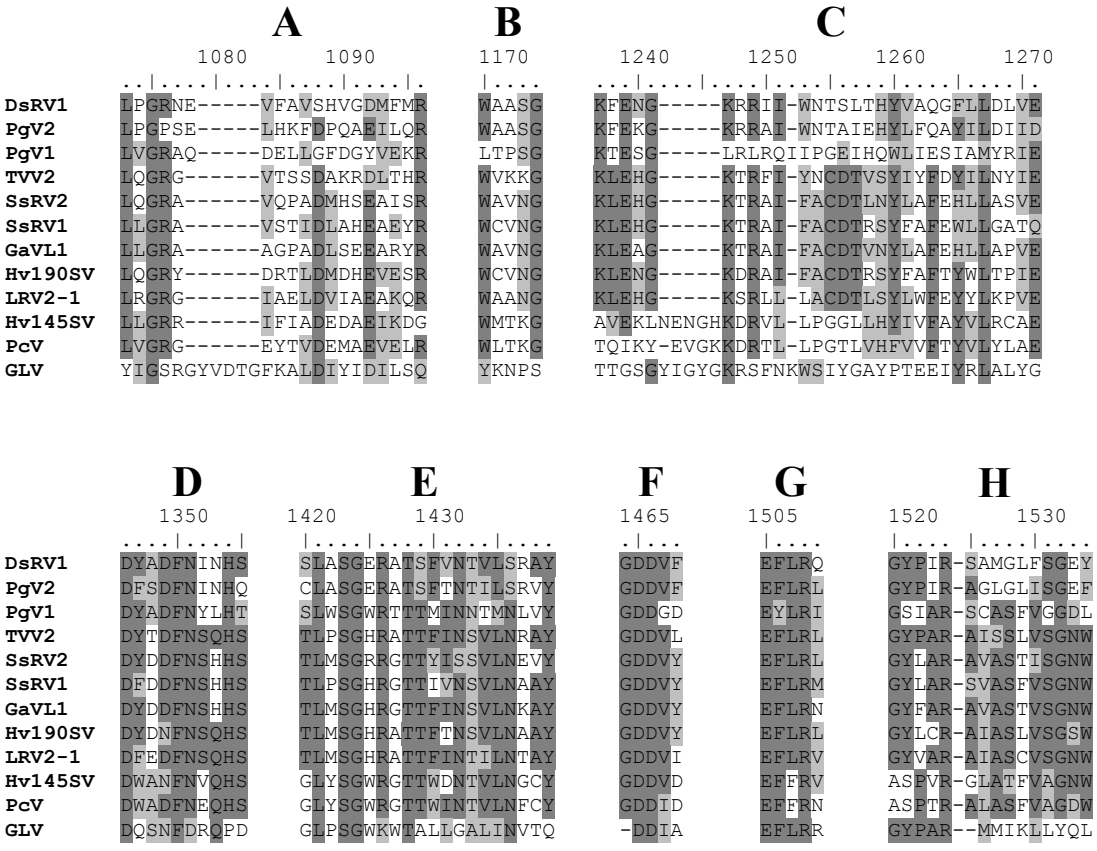


**Figure 5.** The most parsimonious phylogram generated after a phylogenetic analysis of the amino acid sequences of the RdRp genes of DsRV1 (EU547739) compared to viruses of the *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridae*, *Reoviridae* and the genus Endornavirus. The cucurbit yellows-associated virus (CYV) (CAA63099), a (+) ssRNA plant virus was used as out-group. Numbers on the nodes of the tree represent bootstrap values.