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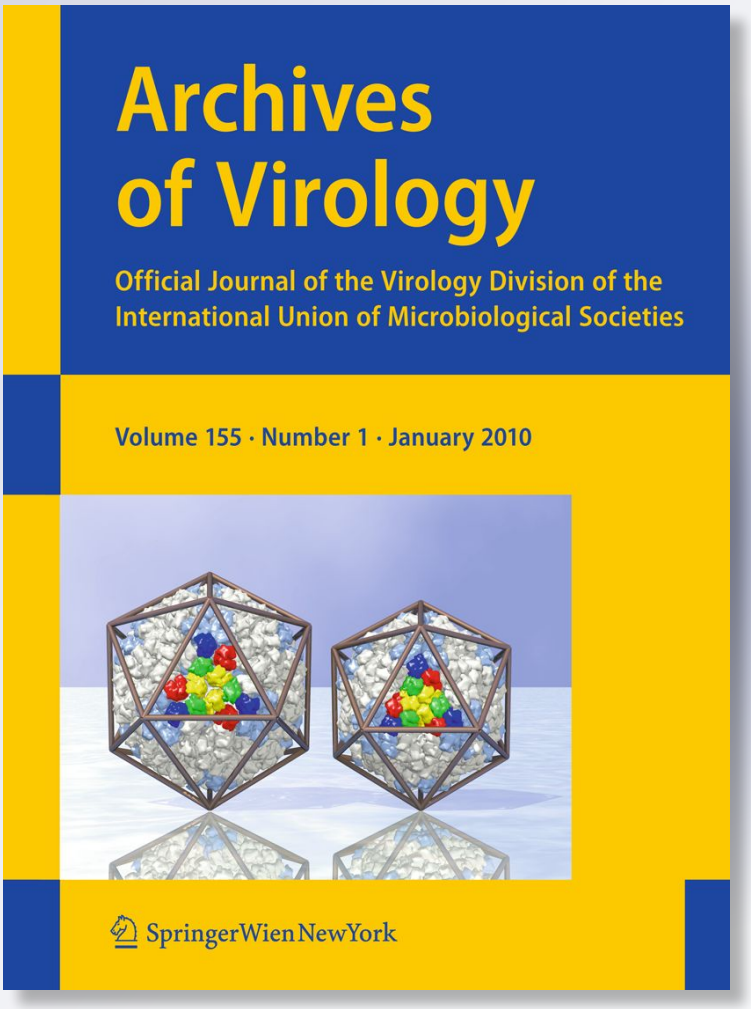

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ORIGINAL ARTICLE

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 dieback 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 been characterized previously. 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 to 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 degree of similarity to proteins of the vacuolar protein-sorting (VPS) machinery, and the other for an RNA-dependent RNA polymerase (RdRp). Phylogenetic comparisons based on amino acid sequence 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 high sequence identity with SsRV1 or SsRV2, and they probably have different recent evolutionary origins.

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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 dieback, 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 its distribution restricted to the northern hemisphere except for a recent report in South Africa [2]. However, *D. pinea* can be highly virulent and has a worldwide distribution [4, 7–9]. Phylogenetic analysis using DNA sequences of protein-coding genes and microsatellite markers has 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 an 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*, 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 subculture, 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, The 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 were used to extract dsRNA from the mycelium (1 ml 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 of isopropanol and 0.1 volumes of sodium acetate. The dsRNA was recovered by centrifugation for 30 min at 13 000 rpm at 4°C, washed with 70% ethanol, dried, and resuspended in 50 µl DEPC-treated distilled water. The isolated dsRNA was separated on a 1% agarose (w/v) gel (Promega) stained with ethidium bromide, using a 1× 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 a 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 a Roche cDNA synthesis kit (Roche Diagnostics, Germany). The dsRNA and random hexamer primers were denatured for 10 min at 99°C, followed by the first- and second-strand syntheses, which were done following the manufacturer's instructions. The synthesized dsDNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, GmbH, Germany) and cloned using a Lucigen PCR-SMART non-proofreader cloning kit (Lucigen® Corporation, Middleton, WI, USA). PCR-amplified inserts were purified using a Roche PCR

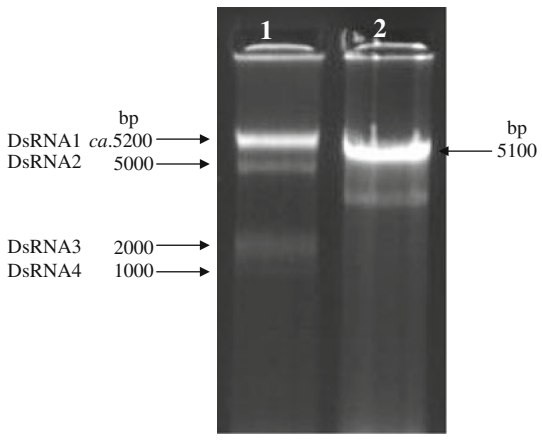

Fig. 1 A 1% agarose gel showing the dsRNA segments isolated from *D. scrobiculata* (lane 1) compared to SsRV1 and SsRV2 isolated from *D. pinea* (lane 2)

Product Purification Kit (Roche Diagnostics, Germany) and sequenced.

Amplification and cloning of the complete viral genome

The randomly amplified cDNA fragments were aligned according to the RdRp gene of Trichomonas vaginalis virus 2 (TVV2), as they shared homology, and genome-specific primers were designed. Sequences between the cDNA fragments were obtained by RT-PCR with genome-specific primers using the Roche Titan One Tube RT-PCR system (Roche Diagnostics, Germany). The 50-µl reaction mixture contained 1× 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 µM each primer and the dsRNA template. The primers and dsRNA were first 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 for 1 cycle at 94°C, 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 a Roche PCR product purification kit and sequenced. Nonspecific RT-PCR products were gel-purified using a PCR product purification kit (Roche Diagnostics, Germany) and ligated overnight to the pGEM-T Easy Vector System II (Promega Corporation, Madison, WI, USA). *Escherichia coli* JM109 cells (Promega Corporation, Madison, WI, USA) were transformed with ligated plasmids 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 a 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'-GCAT TCGACCCGGGTT-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'-AACCCGGGTCGT ATGC-3') complementary to PC4 with a Fermentas First Strand cDNA Synthesis Kit (Fermentas Life Sciences). This was followed by amplification of the cDNA using genome-specific primers and PC5. The products obtained were cloned using the pGEM-T Easy Vector System II, and 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 a 1.5-ml Eppendorf tube for one week at 25°C. After centrifugation, the mycelial pellet was homogenized using a Retsch MM301 homogenizer (30 Hz, 30 s), followed by the extraction of DNA using the technique described by Raeder and Broda [39]. 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'-CC CTAACCTGCGACCTCCGTCG-3') (nt 164) and RDR28 (5'-CCGCCATTTCCTGGGGAAAGGCC-3') (nt 1226) for ORF1 and RDF11 (5'-CCCCGGTAGGAACGAGGTCTT CGC-3') (nt 2180) and RDR2 (5'-CGATACCGTGCATA CCGTAGAACT-3') (nt 3309) for ORF2. As positive controls, the internally transcribed spacer (ITS) regions 1 and 2, the 5.8S ribosomal subunit [46], and dilutions of RT-PCR products obtained from the dsRNA with the same primers

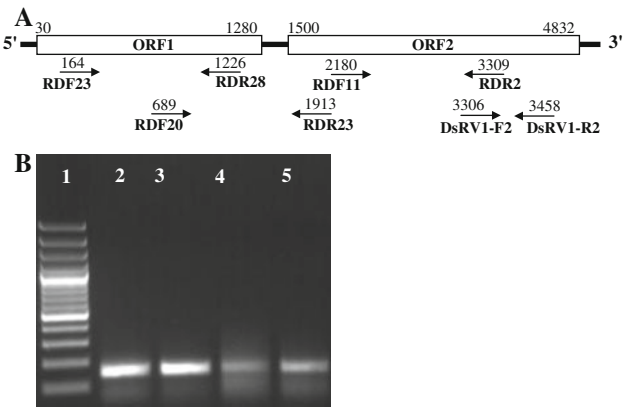

Fig. 2 (**a**) A schematic representation of the genome organization of DsRV1. The white blocks represent the coding regions, and the black blocks represent the untranslated regions. ORF1- and ORF2-specific primers are indicated with arrows in the direction of amplification. The position of the primers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

were amplified. The 25-µl reaction mixture consisted of 10× 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 a 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 an 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 those of 31 viruses belonging to the families *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* and *Endornaviridae* (Table 1). These represent the virus families in which dsRNA mycoviruses have been reported. A positive-sense ssRNA virus belonging to the family *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

Extraction of dsRNA from *D. scrobiculata* mycelium and separation 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), suggested 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'-GGAGATC ACTTCGCTGTACC-3') (nt 689) and RDR23 (5'-GGCA GCAGCCGCCTCCACGG-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 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 [28], as it had 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 RdRps of most dsRNA viruses [5]. 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

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

Virus name	Acronym	Accession number	Reference sequence	Family/genus
Aspergillus ochraceous virus	AoV	ABV30675	–	*Partitiviridae*
Black raspberry cryptic virus	BRCV	ABU55400	–	*Partitiviridae*
Botryotinia fuckeliana totivirus	BfV	CAM33265	–	*Totiviridae*
Cryphonectria hypovirus 1	CHV1	–	NP_041091	*Hypoviridae*
Cryphonectria hypovirus 1-EP713	CHV1-EP	Q04350	–	*Hypoviridae*
Cucurbit yellows-associated virus	CYV	CAA63099	–	*Potyviridae*
Diplodia scrobiculata RNA virus 1	DsRV1	EU547739	–	Unassigned
Fusarium graminearum virus-DK21	FgV-DK21	YP223920	–	Unassigned
Giardia lamblia virus	GLV	–	NP_620070	*Totiviridae*
Gremmeniella abietina RNA virus L1	GaV-L1	–	NP_624332	*Totiviridae*
Gremmeniella abietina RNA virus L2	GaV-L2	YP044807	–	*Totiviridae*
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	*Totiviridae*
Leishmania RNA virus 2-1	LRV2-1	–	NP_041191	*Totiviridae*
Mycoreovirus-1/*Cryphonectria parasitica* 9B21	MYRV1/Cp9B21	BAD51414	–	*Reoviridae*
Mycoreovirus-3/*Rosellinia necatrix* W370	MYRV3/RnW370	YP392478	–	*Reoviridae*
Operophtera brumata reovirus	ObRV	ABB17205	–	*Reoviridae*
Ophiostoma minus virus	OmV	CAJ34336	–	*Totiviridae*
Ophiostoma partitivirus 1	OPV1	CAJ31886	–	*Partitiviridae*
Ophiostoma quercus partitivirus	OqPV	CAJ34337	–	*Partitiviridae*
Penicillium chrysogenum virus	PcV	YP392482	–	*Chrysoviridae*
Phlebiopsis gigantea dsRNA 1	PgV1	CAJ34333	–	Unassigned
Phlebiopsis gigantea dsRNA 2	PgV2	CAJ34335	–	Unassigned
Phytophthora endornavirus	PEV	YP241110	–	*Endornavirus*
Sphaeropsis sapinea RNA virus 1	SsRV1	–	NP_047558	*Totiviridae*
Sphaeropsis sapinea RNA virus 2	SsRV2	–	NP_047560	*Totiviridae*
Trichomonas vaginalis virus 2	TVV2	AF127178	–	*Totiviridae*
Vicia cryptic virus	VCV	ABN71234	–	*Partitiviridae*

the first (nt. 1319) and second methionine (nt 1473) after the stop codon of ORF1, as it has purines at positions -3 and +1 (AAA**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 families *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* and *Endornaviridae* (Fig. 5). DsRV1 grouped with Phlebiopsis gigantea mycovirus dsRNA element 2 (PgV2) and 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 family *Chrysoviridae*, while PgV1

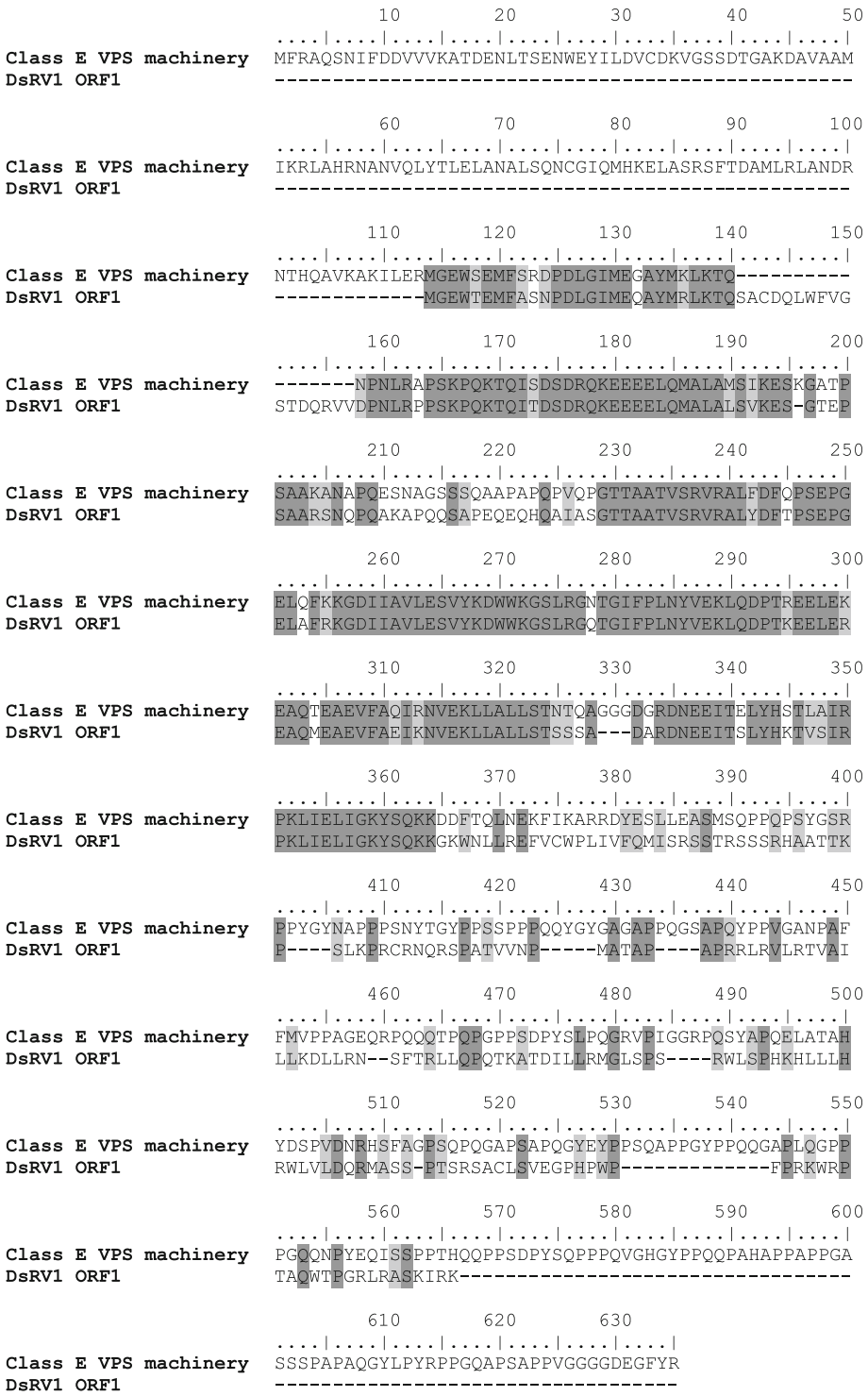

Fig. 3 Amino acid alignments of the complete ORF1 of DsRV1 (EU547739) and a protein belonging to the class E vacuolar protein-sorting (VPS) machinery (Q0U6X7). Dark shading indicates identical amino acids, and lighter shading indicates 60 % amino acid similarity

and PgV2 have not yet been classified. All of 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 families *Chrysoviridae*, *Totiviridae*, *Hypoviridae* and those of the genus *Endornavirus*. The other clade included viruses of the family *Partitiviridae* and the genus *Mycoreovirus*. Viruses belonging to three genera of the family *Totiviridae*, i.e., *Totivirus*, *Leishmaniavirus* and *Giardiavirus*, grouped accordingly, except for Giardia lamblia virus (genus *Giardiavirus*), which was more closely related

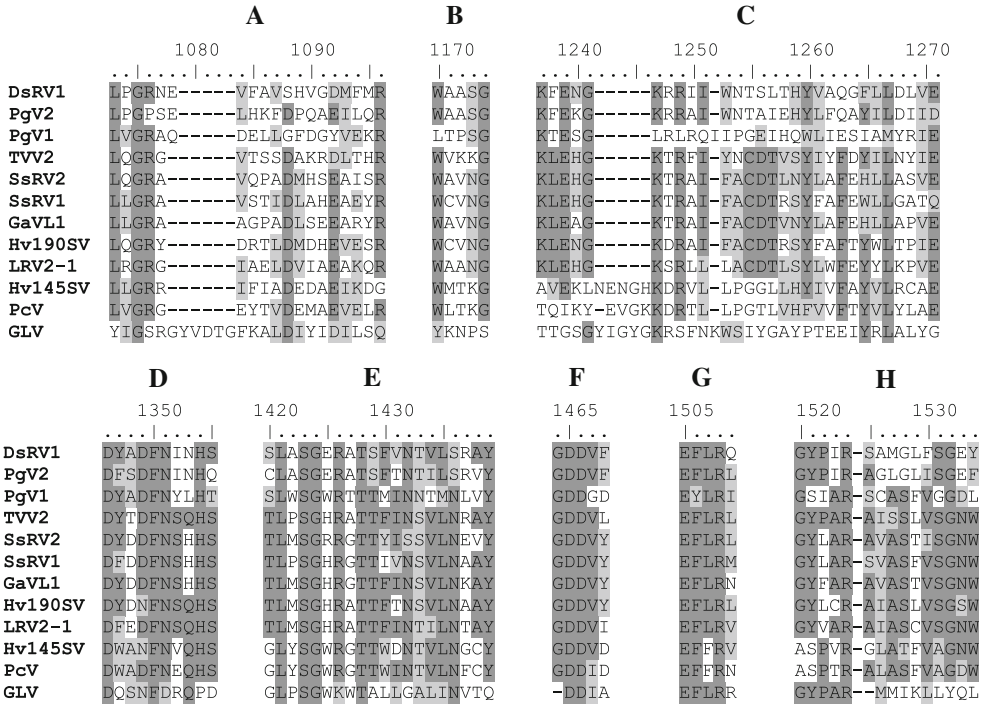

Fig. 4 Partial amino acid alignments of the RdRp genes for a set of dsRNA viruses, showing the eight conserved motifs (marked A-H). Viruses included here are members or proposed members of the family *Totiviridae*, except PcV and Hv145SV, which belong to the family *Chrysoviridae*, and DsRV1, PgV1 and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

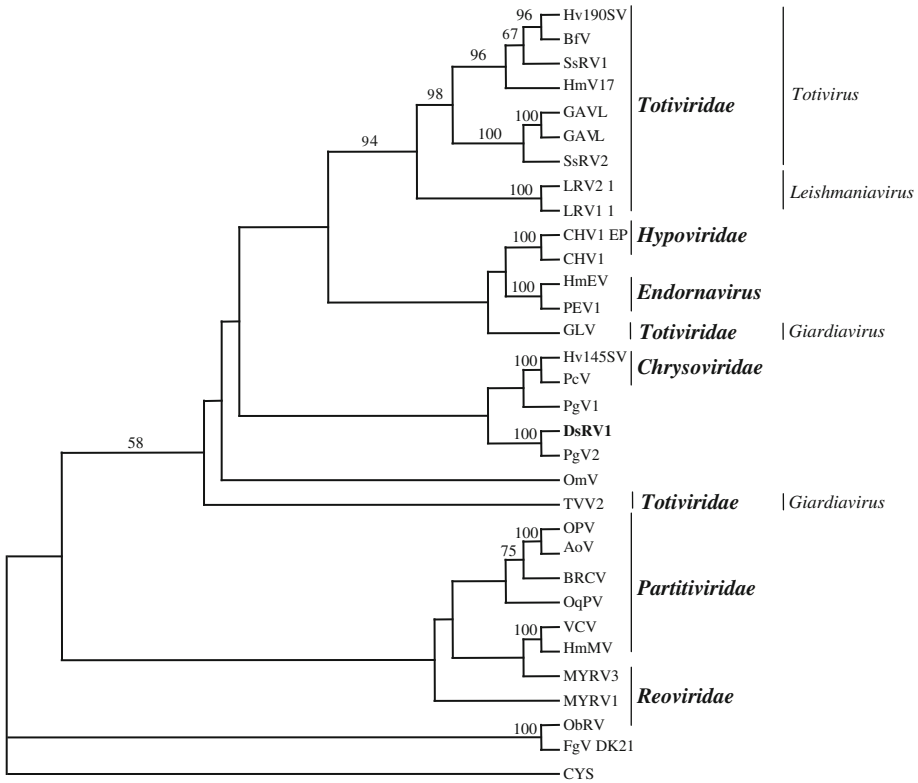

Fig. 5 The most parsimonious phylogram generated after a phylogenetic analysis of the amino acid sequences of the RdRp genes of DsRV1 (EU547739) compared to viruses of the families *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridae*, and *Reoviridae* and the genus *Endornavirus*. Cucurbit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

to viruses of the family *Hypoviridae* and the genus *Endornavirus* than to those of the other two genera (*Totivirus* and *Leishmaniavirus*) in the same family. The mycoreoviruses grouped separately from the insect reovirus included in this study, Operophtera brumata reovirus.

Discussion

The genome of a dsRNA element commonly associated with *D. scrobiculata* was sequenced and characterized in this 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, deletion mutants or degradation products of the largest fragment. Phylogenetically, DsRV1 grouped most closely to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2). Its next-closest relatives are viruses belonging to the family *Chrysoviridae* (Hv145sV and PcV) [18, 25].

DsRV1 was isolated from a Californian *D. scrobiculata* isolate, has a genome size of 5018 bp, and contains 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 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 could be to assist in the formation of subcellular 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 to be present in 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 an equivalent polypeptide.

DsRV1 probably obtained a VSP-like protein from its host, which 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 for RNA viruses: for example, a ubiquitin-coding gene from a togavirus [33] and a putative UDP glycosyltransferase 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 family *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 dsRNAs associated with vesicles are characteristic of a replicative intermediate of an ssRNA virus [24]. DsRV1 and other unencapsulated mycoviruses, therefore, probably had an ssRNA progenitor.

Based on the RdRp (ORF2) sequence, 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. The closest relatives to DsRV1 and PgV2 are another dsRNA element from *P. gigantea* (PgV1) and viruses belonging to the family *Chrysoviridae* (Hv145SV and PcV) [18, 25]. The family *Chrysoviridae* represents a new family established to accommodate mycoviruses with multipartite dsRNA genomes of three to four segments [19] that were previously considered to be part of the genus *Chrysovirus* in the family *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, which 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 diversity, 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.

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