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Molecular characterization of a new monopartite dsRNA mycovirus from mycorrhizal *Thelephora terrestris* (Ehrh.) and its detection in soil oribatid mites (Acari: Oribatida)

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ABSTRACT

A novel dsRNA virus was identified in the mycorrhizal fungus *Thelephora terrestris* (Ehrh.) and sequenced. This virus, named Thelephora terrestris virus 1 (TtV1), contains two reading frames in different frames but with the possibility that ORF2 could be translated as a fusion polyprotein after ribosomal -1 frameshifting. Picornavirus 2A-like motif, nudix hydrolase, phytoreovirus S7, and RdRp domains were found in a unique arrangement on the polyprotein. A new genus named Phlegivirus and containing TtV1, PgLV1, RfV1 and LeV is therefore proposed. Twenty species of oribatid mites were identified in soil material in the vicinity of *T. terrestris*. TtV1 was detected in large amounts in *Steganacarus (Tropacarus) carinatus* (C.L. Koch, 1841) and in much smaller amounts in *Nothrus silvestris* (Nicolet). This is the first description of mycovirus presence in oribatid mites.

Introduction

Mycorrhizal symbioses are fundamental to tree growth, increasing the availability of water and mineral nutrients through extramatrical mycelia (Smith and Reed, 1997). *Thelephora terrestris* (Ehrh.) (earthfan fungus; family *Thelephoraceae* Chevall.) is a pioneer mycobiont which occurs in a wide variety of soils, including both non-fertilized and highly fertilized nursery soils, as well as mineral or peaty soils (Colpaert, 1999). *Pinus* spp. are the most frequent hosts of *T. terrestris*, but the fungus can form mycorrhizas with a wide range of plant genera including alder (*Alnus*), birch (*Betula*), chestnut (*Castanea*), beech (*Fagus*), poplar (*Populus*), oak (*Quercus*), willows (*Salix*), and others (Colpaert, 1999). More than 21% of screened roots of a Norway spruce (*Picea abies*) forest were colonized with *T. terrestris* (Johnsson et al., 1999). Cytokinins of the zeatin riboside-type produced by the fungus are enhanced by the presence of host roots (Kraigher et al., 1991). Furthermore, cytokinins have been held to influence the morphological characteristics and distribution of mycorrhizal roots, resistance to root pathogens, and longevity of roots and seedlings. The presence of *T. terrestris* has been observed to be highly correlated with presence of copper (Cu) and iron (Fe) as foliar nutrients (Smaill and Walbert, 2013), and a high content of aluminum has indicated bioconcentration of this trace element by the fungus (Rudawska and Leski, 2005). *T. terrestris* has been observed to enhance the growth of Japanese poplar (*Populus maximowiczii*) seedlings in an N-deficient substrate (Obase et al., 2009). The fungus is described as tolerant of high nitrogen availability, and therefore it has recently become a common ectomycorrhizal symbiont in conifer tree nurseries across the world.

The exploration of viruses in nonpathogenic mycorrhizal fungi has been widely neglected, and little information has been obtained regarding such fungi due to their obligate biotrophic nature. The presence of viruses in fungi should not be surprising, however, as diverse viruses have been described in *Tuber aestivum* and Tuber *excavatum* ectomycorrhizal fungi: *T. aestivum* mitovirus (Stielow et al., 2011a), *T. excavatum* mitovirus (Stielow et al., 2012), *Tuber aestivum* endornavirus (Stielow et al., 2011b), and *T. aestivum*

Abbreviations: PgLV, *Phlebiopsis gigantea* large virus; LeV, Lentinula edodes virus; RfV, Rhizoctonia fumigata virus; NUDIX hydrolase, a hydrolase acting upon a substrate of general nucleoside diphosphate structure linked to another moiety

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virus 1 (totivirus) (Stielow and Menzel, 2010). A unique mitovirus has been described in *Rhizophagus clarus* arbuscular mycorrhizal fungus (Kitahara et al., 2015), and a 4.5 kbp dsRNA element (GRF1V-M) was found in a *Glomus* sp. fungus (Ikeda et al., 2012)

Mites of the suborder Oribatida comprise one of the most diverse and abundant arthropod groups in the upper layers of soil and litter in natural and agricultural systems. Most species are generalists that feed on decomposing plant debris and fungi (Schneider et al., 2004), while others feed on lichens or soil algae (Seyd and Seaward, 1984). They are dominant in forest ecosystems but they occur worldwide in any type of soil, where they are important decomposers. Microphytophagous and panphytophagous species may be important short-distance dispersers of fungal spores, which could be dispersed on the body surface or in the digestive tract of oribatid mites (Renker et al., 2005).

As true of other organisms, mites may be subject to viral disease. Surprisingly, however, only in the citrus red mite, *Panonychus citri*, the European red mite, *P. ulmi*, (both Order Prostigmata, Family Tetranychidae), and an ectoparasitic mite of honeybees *Varroa destructor* (order Mesostigmata, Varroidae) are devastating viruses known in more detail (Van Der Geest et al., 2000; Yue and Genersch, 2005). The presence of other viruses has been documented by microscopy examination where virus-like particles of different sizes have been observed in the predatory gamasid mites *Metaseiulus occidentalis, Neoseiulus cucumeris*, and *Phytoseiulus persimilis* (all Mesostigmata, Phytoseiidae) (Poinar and Poinar, 1998). Mites are also involved in the transmission of viruses. The phytophagous mites *Aceria tulipae* (Prostigmata, Eriophyidae), *Brevipalpus phoenicis*, and *Brevipalpus californicus* (Prostigmata, Tenuipalpidae) are known to transmit plant viruses. *A. tulipae* transmits the *Garlic mite-borne filamentous virus* (Allexivirus) (Van Dijk and Van der Vlugt, 1994), while rhabdovirus-like viruses have been transmitted by *B. phoenicis* and *B. californicus* (Kitajima et al., 2003; Kondo et al., 2003; Rodrigues et al., 2003). *West Nile virus*, *Fowl pox virus*, and *Tick-borne encephalitis virus* have been isolated from mites of the superfamily Dermanyssoidea (Mesostigmata), which may play an important role in the transmission of these viruses (Moro et al., 2005; Van der Geest, 2010).

In addition to the pathogenic effects of viruses on higher life forms, both the role of viruses in wild host populations and details about vector–virus–host relationships pointing to long-standing interactions among partners remain fragmentary (Roossinck, 2015). In this paper, we describe a new virus found in the mycorrhizal fungus *T. terrestris* and its presence in *Steganacarus (Tropacarus) carinatus* oribatid mite from the vicinity of that fungus.

Results

Description of the T. terrestris virus 1 genome

In dsRNA isolation from asymptomatic fruiting bodies of *T. terrestris* fungus, which shared 100% nucleotide identity with the *T. terrestris* voucher BB64_301_Oh_Pi_150506 18 S rRNA gene, GenBank AC:HM189958.1, a single band of about 10 kbp was visible on agarose gel (Fig. 1).

A single contig 10,316 nt long was assembled and found to contain two open reading frames (ORFs) in different frames on the genomic plus strand: ORF1 in frame 2 encodes a 202 kDa (1843 aa) protein and ORF2 in frame 1 encodes a 174 kDa (1548 aa) protein. Similarly as in *Phlebiopsis gigantea* large virus 1 (PgLV1) and 2 (Kozlakidis et al., 2009), there are no in-frame stop codons upstream from the ORF2 start codon for 195 nt but ribosomal -1 frameshifting sequence AAAAAAA was found at nt 5492–5498 (56 nt upstream from the stop codon of ORF1). This raises the possibility that ORF2 could be translated as a fusion protein with ORF1 in similar way as it was described in Rosellinia necatrix megabirnavirus 1 and its host *Cryphonectria parasitica* (Salaipeth et al. 2014). In contrast to PgLV1, no pseudoknot structure was predicted downstream of the shifty site in the HPknotter program (Huang et al., 2005), but stable stem-loop structures (results not shown) that could assist in pausing translating ribosomes were predicted upstream as well as downstream of the slippery site in RNAfold (Gruber et al., 2008). Polyprotein processing is intrinsic for many viruses. In picornaviruses, proteinase $2A^{pro}$ cleaves between 2A and the N-terminal proline of 2B or the C-terminal NPGP motif of 2A promotes ribosome skipping during translation (Palmenberg et al., 2010). The 2A-like motif comprises the seven aa residues G/DxExNPGP and N-terminal proline of 2B protein (underlined). These sequences had been found also in insect ssRNA iflaviruses, tetraviruses, and dicistroviruses, in dsRNA rotaviruses, cypoviruses, and in non-fungal totiviruses (Donnelly et al., 2001; Nibert, 2007). In our PSI-BLAST screening of the GenBank database, the motif was found in mycoviruses Rosellinia necatrix mycovirus 2-W 1032/S6 (Alphapartitivirus), Fusarium graminearum hypoviruses 1 and 2 (FgHV1 and 2, Hypoviridae), PgLV1, as well as the newly sequenced virus from *T. terrestris* (TtV1) (Fig. 2a). In Rosellinia necatrix mycovirus 2-W 1032/S6, the 2A-like motif was found after the RdRp domain, in FgHV1 twice (at aa position 749 and 879, after putative peptidase C7), in FgHV2 once at position 242 and once in PgLV1, and in TtV1 close to the N-terminus of ORF1 (Fig. 1). Based on expected activity of $2A^{Pro}$, the N-terminal part of TtV1 polyprotein 90 aa long could be released from the polyprotein.

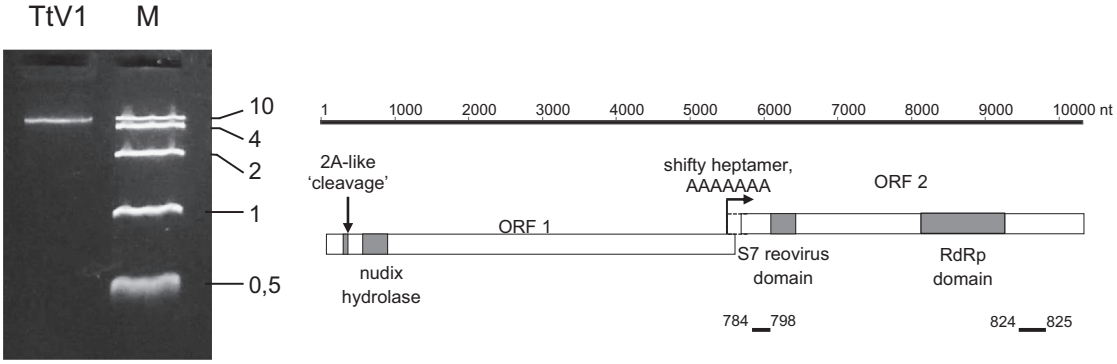

Fig. 1. Agarose gel electrophoresis of dsRNA isolated from *T. terrestris*, analysis of nucleic acids. M –DNA Ladder (10 kbp, 4 kbp 2 kbp, 1 kbp, 500 bp). Genome arrangement and motifs of TtV1. Schematic representation of the genomic organization of TtV1 shows the presence of two ORFs (ORF1 and ORF2). The dotted-line box indicates a possible extension of ORF1 via a translational frameshift mechanism. Position of 2A-like motif, nudix hydrolase, S7 phytoreovirus domain, and RdRp conserved domain are indicated by boxes. Regions used for RT-PCR detection with primer numbers are indicated by thick lines.

a

Virus	AC	
TtV1		V K A D C V G L V G V E T N P G P
PgLV1	YP_003541122.1	A L A D N I G L V G I E T N P G P
Rosellinia necatrix mycovirus 2-W1032/S6	BAM36407	P K R D L T V D G D V E K N P G P
FgHV1gp2	YP_009011065	V I A R N A D D A D V E L N P G P
FgHV1gp2	YP_009011065	V D P Y G A D D H D V E K N P G P
FgHV2/JS16	YP_009130646	K R A R D S S M A D I E P N P G P
Consensus mycoviruses		G/D V/I E N P G P

Consensus positions are highlighted in gray.

b

Virus	AC	
TtV1		G V G G S A E K D E S P S D T L T R E A K E E V G I D I
PgLV1	YP_003541122.1	G V G G S L E L G E D P S A G I A R E V R E E V G I D I
LeV1 -HKB	BAG71789.2	G I G G K I D S R E D L I A G L N R R V M E E V S I D I
RfV1	AJE29744	F P G G K V D A G E S I Q D A L K R E V M E E T G L T V
Consensus mycoviruses		* * * * * *

Conserved positions are marked with asterisk, consensus amino acid positions of nudix motif are highlighted in gray.

Fig. 2. a. Comparison of the amino acid sequences around 2A-like motifs in mycoviruses. b. Nudix hydrolase motif in mycoviruses. Consensus positions are highlighted in gray. Conserved positions are marked with asterisk, consensus amino acid positions of nudix motif are highlighted in gray.

Table 1
Locations and environs of *Steganacarus (Tropacarus) carinatus* mites.

sample no:	identification in collection	locality	collection year	Environs	PCR 824+825	PCR 784+817
5	163-7-e/17/CZ391	49°05′52″N 14°08′36″E	1997	Birch forest	+++	+++
6	164-6-f/2/CZ392	49°04′05″N 14°11′17″E	1997	Old oak forest	+++	+++
9	216-2-f/1/CZ1318	50°42′06″N 14°51′22″E	2002	Acidophilic spruce grove	+++	+++
13	240-10-c/1/CZ3245	49°53′39″N 13°20′35″E	2009	Clearing	+	++
15	242-2-c/1/CZ3276	49°17′41″N 13°41′29″E	2009	Hazel tree, spruce, willow	+++	+++
20	326-9-a/2/CZ-4406	50°11′21″N 14°43′57″E	2010	Young pine forest, linden tree	+	–
21	327-7-a/1/CZ4359	50°20′48″N 15°04′33″E	2010	Larch, spruce, oak	+++	+++
23	205-3-d/1/SK69	49°04′ 40″N 22°25′29″E	1999	Beech forest	+	+++

ns – not sequenced

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            I              II                 III                  IV                           V                        VI          VII          VIII
TtV1     LVGRA  79  IAPRGS    52  GLRLRQII   57  DYADFNYLH  66  LWSGWRTTTMINNTMNLVYN  21  NGDD  37  EYLR  13  RTCAS
PgV1     LVGRA  79  VAPRGS    52  GLRLRQII   57  DYADFNYLH  66  LWSGWRTTTMINNTMNLVYN  21  NGDD  37  EYLR  13  RSCAS
LeV1     LSGRA  73  LAPTGA    52  GLRSRQIV   59  DYADFNFLH  65  LWSGWRTTSVINNVFNEVYG  21  NGDD  37  EYTR  13  RGIAS
RfV1     LAGRY  72  VGASGS    50  G-KLRQLL   61  DYADFNITH  65  LWSGWRSTMFFNILFNYLYA  21  MGDD  37  EFLR  12  RSICG
SsMBV1   LGRFY  72  YGSSGS    50  G-KLRQLL   56  DWADFNITH  71  LWSGWRSTQFFNSSFNVAYC  21  AGDD  37  EFLR  14  RSIGS
RnMBV1   LGRFF  72  FGSSGS    50  G-KLRQLL   56  DWADFNITH  67  LWSGWRTTSFINCSFNVAYC  21  AGDD  37  EFLR  14  RSIGS
         *.::.        :..*:       *..:**..        *:****..*       ******:*  .:*. :*  .*       .***      *.:*      *::.:
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Fig. 3. Alignment of the conserved RdRp region for TtV1 and related viruses. Eight motifs (I–VIII) are detected in the sequence of the conserved RdRp region. Identical residues are indicated by asterisks. Conserved and semi-conserved amino acid residues are indicated by colons and dots, respectively.

Nudix hydrolase conserved domain of the 23 aa Gx_5Ex_5[UA]$xREx_2EExGU$, where U is I, V, or L, has been identified within aa position 162-273 of ORF1 of TtV1 by a BLAST search for a conserved domain with *E*-value 2.93e-07. Similar domains have been found in ORF1 of PgLV1 within aa 450–511 (*E*-value 2.3e-08), in ORF1 of LeV-HKB within aa 368–390, and in ORF1 of Rhizoctonia fumigata virus 1 (RfV1) within aa 83–173 (Fig. 2b) (Magae, 2012; Li et al., 2015). This enzyme is widespread among eukaryotes, bacteria, archea, and dsDNA viruses, and it catalyzes the hydrolysis of nucleoside di- and triphosphates with various substrate specificity (McLennan, 2006). Nudix hydrolases are typically small proteins (16–21 kDa) having the so-called "housecleaning" function of clearing the cell of potentially deleterious endogenous metabolites (McLennan, 2006). DsDNA poxviruses encode two related nudix hydrolases putatively involved in mRNA cap inactivation or membrane trafficking during virus assembly (McLennan, 2006). The motifs from TtV1, PgLV1, RfV1 and LeV differ from that of dsDNA viruses and in a phylogenetic analysis they form a distinct cluster (not shown).

Phytoreovirus S7 domain motif has been found upstream of the RdRp domain of TtV1 at aa positions 134–230. This domain has been found in PgLV1 and 2 and in all known chrysoviruses, unclassified Glomus sp. RF1 medium virus, Sclerotinia sclerotiorum nonsegmented virus L (SsNsV-L), Fusarium graminearum dsRNA mycovirus 3, and Diplodia scrobiculata RNA virus (Liu et al., 2012).

No other protease or capsid protein-related motifs were identified in the TtV1 polyproteins. If the Lentinula edodes spherical virus (LeSV) and LeV are variants of a single virus, then a putative CP of about 120 kDa is located in the second part of ORF1 (Won et al., 2013). The aa sequence identity of the ORF1 protein was 44% and 20% with PgLV1 and LeV, respectively. All eight motifs described for RdRp have been localized in ORF2 within aa 804-1189 (Fig. 3).

TtV1 and its putative vectors

Twenty species of oribatid mites have been identified in the uppermost soil layer (Supplementary Table 2), with *Hermannia gibba*, *Nothrus silvestris*, and *Steganacarus (Tropacarus) carinatus* being the species most frequently encountered. A TtV1-specific RT-PCR test was performed with each species. *Steganacarus (Tropacarus) carinatus* was

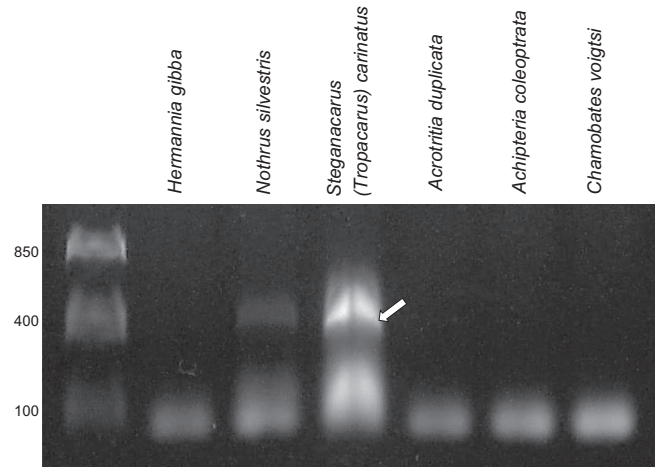

Fig. 4. RT-PCR detection of TtV1 in oribatid mites. Virus-specific amplification product is marked with arrow.

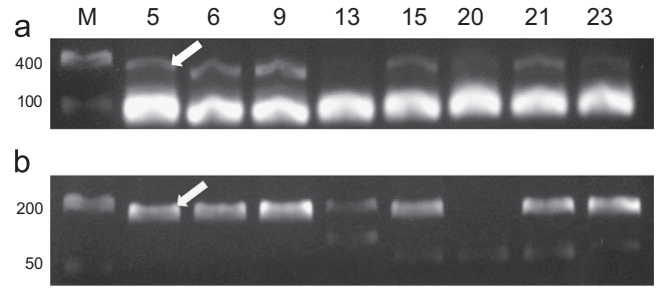

Fig. 5. Detection of TtV1 in populations of *S. (Tropacarus) carinatus.* Amplification with 824 and 825 primers a), amplification with 784 and 798 primers b). Bands of 314 bp, and 148 bp are marked with arrows.

the only sample emitting a significant signal, although a very faint band was visible with the *N. silvestris* sample (Fig. 4). The nature of the band from *S. carinatus* was confirmed by sequencing and comparison with the TtV1 sequence, where 100% identity was observed. We could speculate that the virus was present merely on the surface of *N. silvestris*, whereas, by contrast, the virus was present in the body content of *S. carinatus*.

The presence of TtV1 in different *S. carinatus* populations was tested in individuals collected previously in various types of forest soil in the Czech Republic and Slovakia (Table 1). In PCR with 824 and 825 primers, all but one sample (13) produced a visible amount of product with the expected size of 314 bp (Fig. 5a). In amplification with 784 and 798 primers, sample 13 formed a significantly weaker band than did the other samples and sample 20 failed in this amplification (Fig. 5b). Sequence comparison of the 824 and 825 product showed nt sequence identity of samples from different locations (AC:KT898943).

New genus proposal

An unrooted tree was generated by the method using the MEGA program with amino acid sequences of RdRp genes and 1,000 bootstrap replications. This analysis grouped TtV1 with PgLV1, RfV1, and LeV in a clade distinct from Totiviridae and Chrysoviridae (Fig. 6). These viruses share a similar genome structure with putative frameshift reading and polyprotein expression strategy. The 2A-like motif in the N-terminal part of ORF 1 was identified in the TtV1 and PgLV1 sequences but not in the LeV sequence. The nudix hydrolase sequence was conserved at a similar position in all four viruses, however, and the location of phytoreovirus S7 domain differs from SsNsV-like (Sclerotinia sclerotiorum nonsegmented virus L) and CiTV1-like (Circulifer tenellus virus 1) viruses. We propose establishing a new genus "Phlegivirus" containing PgLV1, TtV1, LeV, and RfV1 viruses. More sequence data is necessary for reliable classification of HmRV-L2 despite its evident close relationship to the new genus.

Discussion

Great efforts to reveal the presence of viruses in cultivated and/or pathogenic fungi in recent decades have resulted in recognition of more than 100 viruses with predominantly dsRNA genome, but non-pathogenic fungi have often been neglected in virus screening. The *T. terrestris* fungus has an ambiguous reputation, being regarded as a weak parasite in some instances because it easily outcompetes other ectomycorrhizal species when inoculated together (Weir 1921, cited in: Colpaert 1999). The complete genome sequencing of TtV1 was performed solely from fruiting bodies of *T. terrestris* from nature. Our attempts to transfer the culture to controlled conditions on agar plates were unsuccessful, as were attempts to isolate the fungus from ectomycorrhizal root tips using the method of Marx et al. (1970). It has been noted that *T. terrestris* spores did not germinate in synthetic media but that they require the presence of a living root of *Pinus sylvestris*, *P. abies*, or other host to germinate (Birraux and Fries, 1981). Fungi growing along the mites' routes on petri dishes were isolated and identified, but none has yet been identified as *T. terrestris*. As no virology research had been done on this fungus to date, we could speculate, in agreement with recent knowledge about mycovirus–fungus etiology, that some physiological states could be virus-influenced.

The effect of viruses on the competitive ability of infected fungi has been studied on *Heterobasidion* spp. infected with HetRV3-ec1 and HetRV6-ab6 and different mycorrhizal and decay fungi (Hyder et al., 2013). That study shows that a single virus strain confers different effects on different *Heterobasidion* host strains and a single virus strain may have contrasting effects on the fitness of a single host isolate (ranging from no effects to harmful or beneficial effects) (Hyder et al., 2013). The GRF1V-M-free culture line of *Glomus* sp. produced twofold greater number of spores and promoted plant growth more efficiently than did the GRF1V-M-positive lines (Ikeda et al., 2013).The TW-2 isolate of *P. gigantea* had been used initially as a biocontrol agent in the United Kingdom, but it gradually lost its efficacy against its target, *Heterobasidion annosum*. Later, the two large viruses PgLV-1 and PgLV2 were found there (Kozlakidis et al., 2009). *P. gigantea* infected with PgLV-1 produced thin layers of mycelial growth and had a powdery-like appearance as compared to the uninfected isolates, which had a denser mycelial growth habit and aerial mycelia above the agar surface. One of the virus-infected isolates was the second-lowest spore producer. Fungal cultures infected with PgV 1 and 2 and Rosellinia necatrix megabirnavirus 1 were hypovirulent (Chiba et al., 2009, Kozlakidis et al., 2009), even though this is not typical for dsRNA in the *Totiviridae,* which appear to have no obvious phenotypic or hypovirulent effects on their hosts (Chiba et al., 2013). Furthermore, in a recent paper, infection of *Sclerotinia sclerotiorum* strains with related SsMBV1 megabirnavirus showed no significant differences in colony morphology, growth rate, and virulence from their original virus-free strains (Wang et al., 2015). In the case of Lentinula edodes mycovirus HKB (LeV) infecting shiitake, the viral dsRNA was present in a strain showing imperfect browning as well as in an asymptomatic strain (Magae, 2012).

The present study revealed that TtV1 has the highest similarity (53% identities) with the previously reported PgLV1 and LeV isolates based on putative RdRp protein (Table 3) and that they are clustered into an independent phylogenetic clade, previously noted as PgRV-

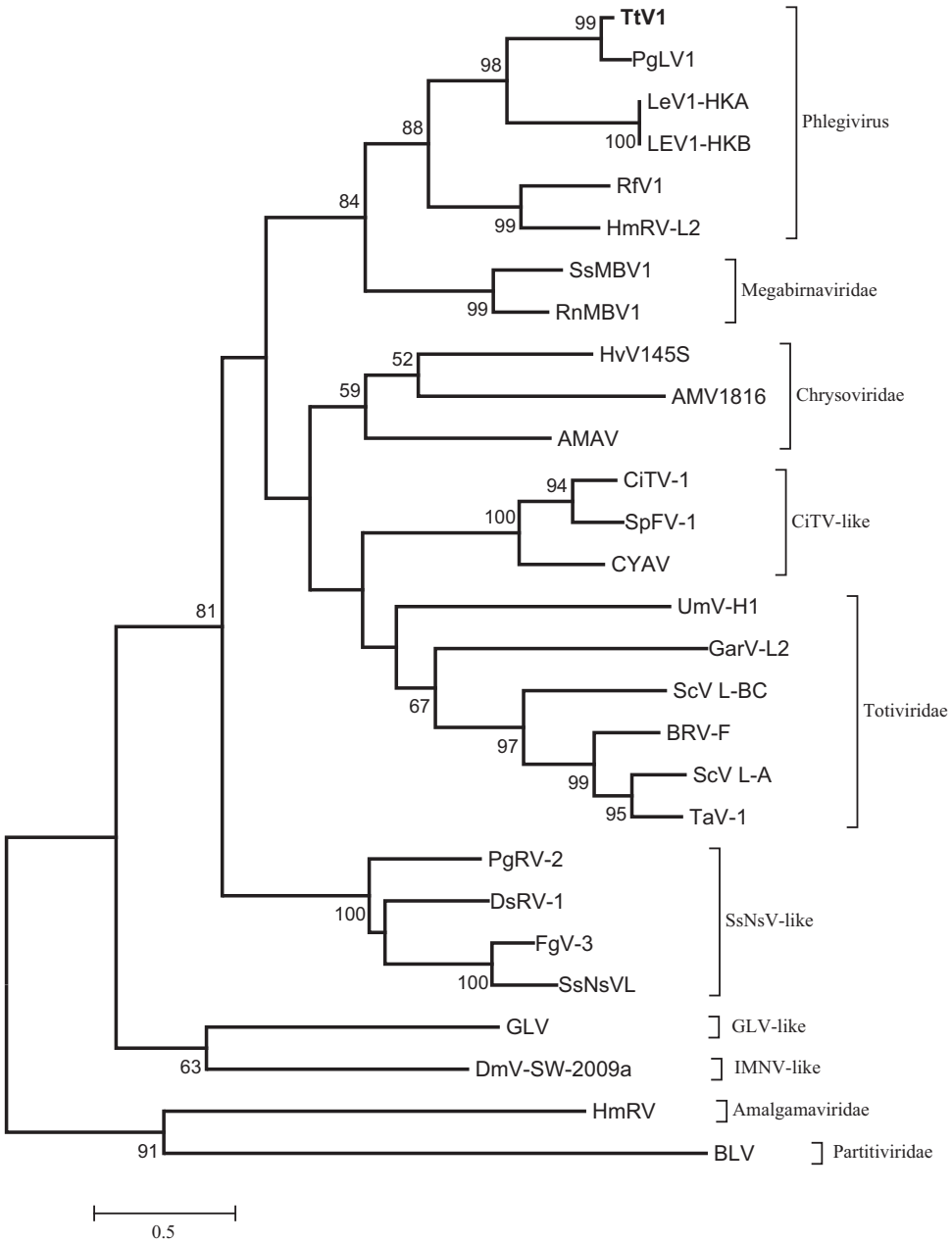

Fig. 6. An unrooted maximum-likelihood tree of species around the proposed genus Phlegivirus. Scale bar corresponds to 0,5 amino acid substitution per site. The tree is based on the amino acid sequences of the region between motif I and VII of the polymerase. Numbers on nodes show bootstrap values above 70 % (1,000 replicates). See supplementary Table 2 for abbreviations of virus names and accession numbers.

like clade (Liu et al., 2012). It is not clear if members of this clade encode for capsid protein gene, as no virion particles associated with PgLV1 have been described (Kozlakidis et al., 2009). LeV HKB was not reported to form virions (Magae, 2012), but the virus named Lentinula edodes spherical virus (LeSV), which is evidently an isolate of the same species as LeV HKB (99% of identity, partial sequence is available, JQ687140), was experimentally confirmed to be encapsidated in spherical particles of 55 nm. Additionally, it was shown that the 120 kDa coat protein originated from the C-terminal part of the ORF1 product (Won et al., 2013). The first two amino acids of the coat protein ("SA") are present in ORF1 of PgLV1 as well as in TtV1 in corresponding positions. As the TtV1 sequence has been obtained from collected environmental samples, purification of its virions must yet be performed in order to resolve this question. 2A-like sequences are present in plus-strand RNA viruses from the families *Picornaviridae*, *Dicistroviridae*, and *Tetraviridae*. In dsRNA viruses, the sequences are found several times in group C rotaviruses (family *Reoviridae*) and infectious myonecrosis virus (tentatively assigned to the family *Totiviridae*) (Nibert, 2007). The presence of such motifs in the ORF suggests that the ORF product is a polyprotein that is cotranslationally "cleaved" into several fragments there. It seems that maturation of the coat protein in TtV1 or PgLV1 involves motifs other than 2A.

Proteins with overly representative numbers of proline, alanine, and serine (PAS-rich proteins) have been encoded by most unclassified dsRNA and unencapsidated in conventional virus particles. The presence of PAS-rich protein (25.9%) was first noted in the ORF1 protein of PgLV1 (Kozlakidis et al., 2009), but its role remains obscure. Similar proportions of P, A, S (22.7%) were noted in ORF1 of LeV-CZ (Kanhayuwa et al., 2015), in RfV1 ORF1 (24.8%), and in TtV1 ORF1 (24.3%), however, thus suggesting some importance of PAS-rich proteins for these viruses.

Table 2
Oribatid mites collected and identified in vicinity of *T. terrestris* with TtV1. Species, where TtV1 was detected are in bold.

No.	Species
1	*Hermannia gibba* (C.L.Koch, 1839)
2	***Nothrus silvestris* Nicolet, 1855**
3	*Microtritia minima* (Berlese, 1904)
4	*Phthiracarus* sp.1
5	***Steganacarus (Tropacarus) carinatus* (C.L.Koch, 1841)**
6	*Acrotritia duplicata* (Grandjean, 1953)
7	*Achipteria coleoptrata* (Linnaeus, 1758)
8	*Chamobates voigtsi* (Oudemans, 1902)
9	*Eupelops torulosus* (C.L.Koch, 1839)
10	*Oribatula tibialis* (Nicolet, 1855)
11	*Hemileius initialis* (Berlese, 1908)
12	*Eueremaeus oblongus* (C.L.Koch, 1835)
13	*Liacarus coracinus* (C.L.Koch, 1841)
14	*Carabodes ornatus* Štorkán, 1925
15	*Banksinoma lanceolata* (Michael, 1885)
16	*Tectocepheus velatus* (Michael, 1880)
17	*Lauroppia falcata* (Paoli 1908)
18	*Suctobelbella subtrigona* (Oudemans 1900)
19	*Suctobelbella subcornigera* (Forsslund 1941)
20	*Phthiracarus* sp.2

Table 3
Pairwise amino acid identity (%) of complete RdRp and ORF1 of viruses related to TtV1.

ORF1	RdRp			
	TtV1	**PgLV1**	**LeV**	**RfV1**
TtV1		53.1	24.3	19.0
PgLV1	44.7		23.2	19.7
LeV	19.9	20.8		20.9
RfV1	12.2	13.4	11.6	

RdRp above diagonal, ORF1 below diagonal.

The role of oribatid mites in virus ecology and especially in TtV1 and *T. terrestris* association is in most aspects unknown. Twenty species of oribatid mites have been isolated from the uppermost 10 cm soil layer within a 1 m diameter from the *T. terrestris* fungus, and only two species (most frequently in mixed and deciduous forest soils) were joined with the TtV1. We could not confirm the presence of *T. terrestris* in all places where different populations of *Steganacarus (Tropacarus) carinatus* mites were collected, as this was not a matter of interest at that time. Nevertheless, TtV1 genome segments were amplified and sequenced in all eight samples. We could speculate either about the ubiquitous presence of *T. terrestris* in the ecosystems, as indicated by Colpaert (1999), and its association with TtV1 or about another widely present but yet unknown soil fungus as a natural host of TtV1.

Based on the sequence analysis presented in this study, we propose the creation of a new genus of name Phlegivirus (acronym from *P. gigantea* host of PgLV1) with PgLV1, LeV1 (with LeSV being an isolate of LeV1), and TtV1 as members. The members of the genus are characterized by unipartite RNA genomes encoding two ORFs with potential ribosomal frame-shifting. Formation of isometric particles was shown at least for LeV1.

Although it is doubtful whether vector mites suffer from the presence of viruses inside their bodies, it has been reported that High plains virus (a synonym of Wheat mosaic virus) and Pigeon pea sterility mosaic virus do seem to replicate in their respective vectors (Kumar et al., 2002). The Brome mosaic virus is capable of multiplying in the eriophyid *Aceria tulipae*, where it may cause severe cytopathological effects in the midgut cells of the mite (Paliwal, 1972). Fungal species from a wide range of taxa (Basidiomycota, Ascomycota, and Zygomycota) have been found to be associated with oribatid mites, thus indicating an important role of these animals for the dispersal of several fungal taxa in soil (Renker et al., 2005).

Conclusions

In this paper, we present the molecular characterization of a new dsRNA monopartite mycovirus isolated from an endosymbiotic fungus; propose creation of the new genus Phlegivirus containing large, monopartite dsRNA viruses; and demonstrate the first detection of a mycovirus in a soil oribatid mite.

Material and methods

Fungus

Fruiting bodies of *T. terrestris* and soil samples from the uppermost 10 cm layer were collected in the Central Bohemian Highlands (Benešov Hills) (49°33′48″ N, 14°15′33″ E) in a pine forest.

DNA was isolated from about 100 mg of fruiting bodies using a DNeasy kit (Macherey Nagel, Germany) according to the manufacturer's protocol and eluted with 50 μl of elution buffer. The identity of the fungus was confirmed by 23 S rDNA internal transcribed spacer sequencing. One microliter of isolated DNA was amplified using ITS1 and ITS4 primers (Supplementary Table 1) in 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C. The product obtained (about 600 bp in length) was spin column purified and Sanger sequenced with ITS4 primer (GATC Biotech AG, Constance, Germany).

dsRNA isolation, PCR and sequencing

DsRNA was isolated from *T. terrestris* fruiting bodies that had been surface sterilized in 5% bleach using CF-11 cellulose protocol as described by De Paulo and Powel (1995). The dsRNA preparation was digested with DNase I and S1 nuclease for 15 min each to eliminate contaminating DNA and ssRNA. The cDNA was prepared with Superscript IV reverse transcriptase (Life Technologies) and tagged random primers-dN6 5′-CGATCGATCATGATGCAATGC-NNNNNN-3′ as described by Xie et al. (2011). The mixture of cDNA library was purified using a gel extraction kit and eluted in distilled water. The cDNA products were then amplified using a specific primer 5′-CGATCGATCATGATGCAATGC-3′ based on tagged random primers-dN6. The amplified dsDNA was spin column purified and eluted in water. The sequencing library was prepared with a KAPA DNA Library Preparation Kit (KAPA Biosystems, Wilmington, MA) and processed using a HiSeq2500 system (Illumina, San Diego, CA) in high-output mode with a TrueSeq SBS sequencing kit v.3 (Illumina, San Diego, CA) by SEQme.eu (Dobříš, Czech Republic).

Bioinformatic analysis

A *de novo* contig assembly was carried out and verified using CLC Genomic Workbench 7.5 (CLCBio, Aarhus, Denmark). The resulting contigs were blastn and blastx aligned with GenBank. A contig representing almost the complete genomic sequence of TtV1 was verified by amplification with specific primers (Supplementary Table 1) and Sanger sequenced. 5′- and 3′-ends were amplified using a 3′/5′RACE kit (Invitrogen, USA). Phylogenetic relationships of the virus were analyzed using MEGA software

(v.4.1) (Tamura et al., 2007). The sequence was deposited in GenBank under accession number KT191297.

Oribatid mite isolation and identification

Oribatid mites were isolated using Berlese–Tullgren photothermo extractors and maintained in 75% ethanol. Collected mites were cleared in 80% lactic acid and determined to species level using a transmission microscope.

Virus detection in oribatid mites

Total RNA was isolated from 5–10 individuals (depending on body size) of distinct oribatid mite species with an RNeasy kit (Macherey Nagel, Germany), eluted from the spin column, and precipitated with 3 volumes of absolute ethanol. cDNA mix was prepared with Superscript IV reverse transcriptase (Life Technologies), 775 reverse primer, and the RNA. Amplification with 774 and 775 primers was performed in a 20 μl reaction volume through 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Fifty- to 100-times diluted PCR product was re-amplified with nested primers 824 and 825 in 20 cycles as described above but with a 60 °C annealing temperature. PCR product was gel-purified and Sanger sequenced with 825 primer. Total RNA was isolated from a single individual of *Steganacarus (Tropacarus) carinatus* mites from different populations (Table 1) with a MagJET plant RNA kit (Thermo Scientific). cDNA was prepared as above and amplified in 40 cycles with 784 and 798 primers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.009.

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