HfMV1 and Another Putative Mycovirus in Central European Populations of *Hymenoscyphus fraxineus*, the Causal Agent of Ash Dieback in Europe

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Summary

The occurrence of putative, doubled-stranded (ds) viral RNA particles was investigated in the decaying ascomycetous fungal pathogen *Hymenoscyphus fraxineus*. In total, 134 isolates obtained from 134 common European ashes (*Fraxinus excelsior* and *Fraxinus angustifolia*) were obtained from three European countries (Austria, Czech Republic and Slovakia). Three different dsRNA bands of *ca* 2.2, 2.5 and 4.5 kb were confirmed in 28.4 % of the examined *H. fraxineus* samples. The dsRNA band of *ca* 2.2 kb was the most frequent, with 21.6 % occurrence, while the *ca* 4.5 kb band was the least abundant, with 15.7 % occurrence. Complementary, Hymenoscyphus fraxineus mitovirus 1 (HfMV1) was recorded through high-throughput sequencing of dsRNA in one Czech isolate and confirmed to occur in all isolates presenting bands of *ca* 2.5 and/or 2.2 kb in size using direct specific retro-transcriptase (RT) PCR. The Czech mitovirus strain contained a single ORF of 2154 nt, encoding an RNA-dependent RNA polymerase (RdRp). These results confirm the presence of HfMV1 in Central Europe and provide evidence of a potential novel mycovirus in *H. fraxineus*.

Keywords: *Chalara fraxinea*, Illumina sequencing, mycoviruses, mitovirus.

Introduction

The ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. (Baral *et al.* 2014) (syn. *Hymenoscyphus pseudoalbidus* Queloz; anamorph *Chalara fraxinea* Kowalski) causes a lethal disease known as ash dieback in Europe. This disease was first identified in Poland (Kowalski, 2006), but it rapidly spread throughout eastern, central and northern Europe. Because no genetic variability has been observed between European *H. fraxineus* and Asian saprophytic fungus *Lambertella albida,* it has been suggested that it likely originated in East Asia (Zhao et al. 2012; Gross et al. 2014a).

Mycoviruses are primarily RNA viruses that infect all major groups of plant pathogenic fungi (Ghabrial and Suzuki, 2009) and are transferred intracellularly through hyphal anastomosis (horizontal transmission) and spores (vertical transmission) (Ghabrial and Suzuki, 2009). Most

mycoviruses produce cryptic infections. However, some mycoviruses identified within fungal genera, i.e., *Botrytis*, *Diaporthe*, *Fusarium*, *Helicobasidium*, *Helminthosporium*, *Ophiostoma*, *Rhizoctonia*, *Rosellinia* and *Sclerotinia*, have harmful consequences on the host organisms (Wu et al. 2007; Preisig et al. 2000; Chu et al. 2002; Ikeda et al. 2004; Ghabrial and Suzuki, 2009; Doherty et al. 2006; Lakshman et al. 1998; Kanematsu et al. 2014; Chiba et al. 2009; Deng et al. 2003; Yu et al. 2010). The best example of a mycovirus with negative effects on the fungal host is *Cryphonectria hypovirus 1* (CHV1), which is currently used as an effective biocontrol agent against *C. parasitica*, the main driver of the chestnut blight in Europe (Anagnostakis and Day, 1979; MacDonald and Fulbright, 1991).

Mycoviruses have been traditionally detected through the isolation of their dsRNA. The size and the number of dsRNA fragments suggest that these molecules might represent mycovirus genomes. In particular, *Mitovirus* (family *Narnaviridae*), the genus of the recently reported Hymenoscyphus fraxineus mitovirus 1 (HfMV1) (Schoebel et al. 2014), only occurs in fungi and has a linear genome of approximately 2.5 kb with no capsid structure. The genome includes one major open reading frame (ORF), which encodes the RNA-dependent RNA polymerase (RdRp) and has a low GC content (approximately 30 %). Mitoviruses are located and translated in the mitochondria, where these organisms typically exist as dsRNA replicative forms (Ghabrial and Suzuki, 2009).

Although mycoviruses have commonly been detected based on dsRNA isolation, the current sequencing and next generation sequencing (NGS) methods, such as Illumina, Roche or Life Technologies platforms, are proving a useful technique for the direct identification of mycoviruses (Al Rwahnih et al. 2011; Vainio et al. 2015; Ezawa et al. 2015; Feldman et al. 2013). Thus, based on previous studies concerning mycoviruses and the need to identify sustainable control methods that might inhibit the development of ash dieback, we conducted a basic investigation complemented with new technologies to describe the occurrence of fungal viruses in *H. fraxineus*. Specifically, the main objectives of the present study was (1) to investigate the occurrence of viral dsRNA particles among the Central European population of *H. fraxineus* and (2) describe potential new viruses.

Materials and Methods

Sampling

In total, 134 isolates of *H. fraxineus* from Austria, Czech Republic and Slovakia were analysed in the present study (Table 1). Forty-three samples were isolated in the laboratory of Forest Pathology in Mendel University in Brno. The other isolates were obtained thanks to the cooperation within COST Action FP1103 (FRAXBACK).

Fungal isolation

The fungus samples were isolated from ash twigs collected in the field and transported to the laboratory. The sterilisation protocol involved incubation in 70 % ethanol (30 s), followed by sodium hypochlorite solution (30 s), 10 % ethanol (30 s) and sterilised distilled water (2 min). Subsequently, the samples were dried on filter paper and transferred to a Petri dish with Malt Extract Agar (MEA) medium (HiMedia, Mumbai, India). When the mycelium was visible, the sample was immediately transferred to AMEA medium (MEA amended with 50 g fresh, frozen or dried *Fraxinus excelsior* leaves, which were removed after autoclaving) (Kirisits et al. 2013).

DsRNA analyses, cDNA synthesis and HfMV1 screening

DsRNA molecules were extracted following a modified version (Botella *et al*. 2015) of the protocol of Morris and Dodds (1979). Although CF-11 cellulose specifically binds to dsRNA, the 38 positive isolations were repeated twice to confirm the results. Furthermore, S1 Nuclease (Thermo Fisher Scientific, Inc.) treatment was conducted to degrade single-stranded RNA (ssRNA); as suggested by the manufacturer. The dsRNA-banding patterns were assessed through electrophoresis (Botella et al. 2015). The sizes of the dsRNA bands were determined through visual comparison with a Gene Ruler 1 kb Plus DNA Ladder (New England Biolabs).

Sequencing of the 2.5 and 2.2 kb bands and GenBank deposition

To confirm the presence of HfMV1 in the 35 isolates with the bands of *ca* 2.2 and/or 2.5 kb, the corresponding total dsRNA was used for the synthesis of first-strand cDNA by direct specific reverse transcription (RT) PCR as performed in Schoebel *et al*. (2014). The amplified region (*ca* 500-bp RT-PCR products) was separated through electrophoresis on 1 % agarose gels (SERVA).

The isolate MeU_1721 was randomly selected among the isolates presenting both 2.5 and 2.2 kb bands to confirm that both bands belong to HfMV1. The total dsRNA was extracted. Each dsRNA band was cut from the gel and purified to subsequently synthesise the corresponding cDNA and perform direct RT-PCR as described above. The corresponding sequences for MeU_1721-band 1 (2.5 kb) and MeU_1721-band 2 (2.2 kb) were deposited in GenBank/NCBI (accession numbers KT809401 and KT809402).

Description of the full-length sequence of HfMV1 within the isolate MEU_1739 using high-throughput sequencing

Among the positive isolates, MeU_1739 (Table 1), which contained a mixture of 3 dsRNA bands of 2.2, 2.5, and 4.5 kb in size (Figure 1), was selected for the determination and sequencing of potential viruses. A 100-200-ng/µl sample of the extracted dsRNA of MeU_1739 was sent to Macrogen Korea for high-throughput sequencing. Illumina HiSeq 2500 sequencing was performed. The subsequent data processing pipeline included the following steps. (1) Sequence quality was checked using fastQC-0.10.1. (2) The adaptors were trimmed the FASTX Toolkit (FASTX-clipper). (3) To obtain unique reads, the FASTX Toolkit (FASTX-collapser) was used. (4) The contigs were created using the Velvet-1.2.10 assembler (Zerbino and Birney, 2008). (5) Subsequently, the libraries were blasted against GenBank/NCBI. (6) The Short Oligonucleotide Alignment Program (SOAP aligner 2.21) (Li et al. 2009) was used for the efficient gapped and ungapped alignment of short oligonucleotides onto reference sequences selected in the Blastn analysis. (6) Sequences were retrieved using the map format in SAMtools (Li et al. 2009). In addition, complete packages, such as the CLC Genomic Workbench 6.5 (CLC Bio), were used for the visualisation of partial evaluation steps.

The NCBI Protein Blast (Blastx) was used to search for similar sequences and conserved domains. The NCBI OrFinder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Geneious Pro 5.1.6 software was used to identify ORFs. The final sequence was deposited in GenBank/NCBI (accession number KT809403).

Results

Occurrence of dsRNA banding patterns and HfMV1

Three dsRNA bands of *ca* 2.2, 2.5 and 4.5 kb were observed in 28.4 % (38 out of 134) of the observed isolates (Table 1). The three different dsRNA bands infected and co-infected different isolates. Thus, the 2.2-kb band was the most abundant, occurring in nearly 21.6 % of the isolates. The other two dsRNA bands were represented in similar amounts: 17.9 % (2.5 kb) and 15.7 % (4.5 kb). All three bands were identified in 40 % of the positive samples, and more than one band was identified in more than 55 % of cases.

A comparison of the dsRNA presence in these three Central European countries revealed that isolates from the Czech Republic (N= 69) more commonly (36.2 %) hosted dsRNA segments compared with Austrian isolates (N= 26) (23.1 %) and Slovak isolates (N= 39) (17.9 %).

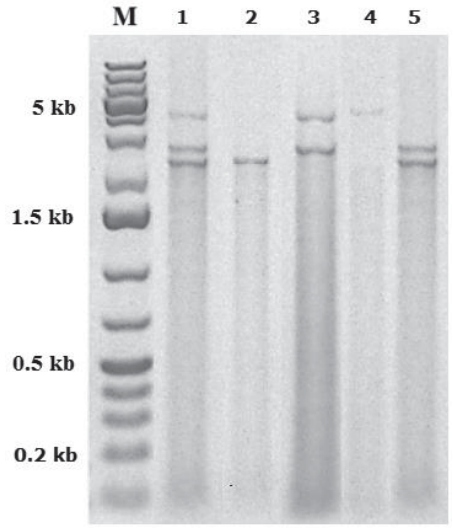

Figure 1. Gel electrophoretic profiles of the dsRNAs (*ca* 2.2 kb, 2.5 kb and 4.5 kb) observed in Central European populations of *H. fraxineus*. M, DNA marker (GeneRuler 1 kb Plus DNA Ladder, 75-20000bp, Thermo Scientific), (1) isolates MeU_1939, (2) MeU_1654, (3) KAR 4, (4) C50/12B, (5) TU 3/2/1/1

Based on direct specific RT-PCR, HfMV1 was confirmed in all the isolates harbouring *ca* 2.2 and/or 2.5 kb-bands. The PCR product was *ca* 500 bp in length, confirming the presence of HfMV1 in Central Europe. Additionally, the 2.2 and 2.5 kb bands belonging to isolates MeU_1721 and MeU_1739, respectively, were processed, and first strand cDNA was synthesised. The direct RT-PCR of the corresponding cDNA using primers specific for HfMV1 confirmed that both bands might belong to the same virus.

Description of the full-length HfMV1 sequence of isolate 1739 using high-throughput sequencing

Among the positive isolates, MeU_1739 (Table 1), which contained a mixture of three dsRNA bands of 2.2, 2.5, and 4.5 kb in size (Figure 1), was selected for the determination and sequencing of potential viruses. Two readings were obtained using Illumina HiSeq 2000 and 100-bp paired-end chemistry. The readings were generated from the end of one read from the sample (after shearing) of either direction. In total, 3,931,685 nucleotides (nt) in raw data were obtained. After quality trimming and sequence unification were completed, 1,738,530 nt were obtained from paired-end reads. The Blastn results showed hits of *H. fraxineus* mitovirus 1 in 22× and hits of *Sclerotinia sclerotiorum* mitovirus 5 in 3×. Based on these results, unique reads were aligned with reference sequences of mitoviruses. The analysis provided a total read count of 27,104 nt and high coverage totalling 1,686 nt of reference sequence (KJ667051) (Schoebel et al. 2014). The resulting consensual sequence was 2350 nt long against 2387 nt

Table 1. Data collection of the *Hymenoscyphus fraxineus* isolates analysed in this study

Code of samples	Year of collection	Locality	Country	dsRNA bands
MeU_1693	2012	Brno - Vojtova street	Czech Republic	(-)
MeU_1694	2012	Brno - Vojtova street	Czech Republic	(-)
MeU_1735	2013	Podolí	Czech Republic	(-)
MeU_1716	2013	Highlands - Třemošnice - Počátky	Czech Republic	(-)
MeU_1723	2013	Seč - Javorka	Czech Republic	(-)
MeU_1705	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1724	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1654	2013	Brno - Ivanovice	Czech Republic	2.2kb
MeU_1721	2013	Highlands Seč Javorka	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1737	2013	Podolí	Czech Republic	2.2kb
MeU_1704	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1701	2013	Vranovice	Czech Republic	2.2kb, 4.5kb
MeU_1653	2013	Brno - Havelková street	Czech Republic	(-)
MeU_1730	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1732	2013	Lužické hory	Czech Republic	(-)
MeU_1738	2013	Podolí	Czech Republic	(-)
MeU_1657	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1665	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1720	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1656	2012	Brno - Ústřední hřbitov	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1702	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1714	2013	Highlands - Třemošnice - Počátky	Czech Republic	(-)
MeU_1734	2013	Podolí	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1731	2013	Lužické hory	Czech Republic	(-)
MeU_1699	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1657	2012	Brno - Ústřední hřbitov	Czech Republic	(-)
MeU_1700	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1698	2013	Vranovice	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1718	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1739	2013	Podolí	Czech Republic	2.2kb, 2.5kb, 4.5kb
MeU_1712	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.2kb, 4.5kb
MeU_1713	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.2kb
MeU_1711	2013	Highlands - Třemošnice - Počátky	Czech Republic	2.5kb
MeU_1696	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1726	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_1703	2013	Highlands - Běštvina - Hlubošský rybník	Czech Republic	(-)
MeU_1733	2013	Lužické hory	Czech Republic	(-)
MeU_1743	2013	Lužické hory	Czech Republic	2.2kb
MeU_1725	2013	Highlands - Seč Javorka	Czech Republic	2.2kb
MeU_1742	2013	Lužické hory	Czech Republic	2.2kb
MeU_1652	2012	Brno - Bohunice, ul Havelková	Czech Republic	(-)
MeU_1729	2013	Highlands - Seč Javorka	Czech Republic	(-)
MeU_13	2013	Vranovice	Czech Republic	(-)
C54/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C52/12A	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C46/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	(-)
C75/12B	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C8/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	2.2 kb
C106/13A	2013	Dolní Světlá - Liberecký kraj	Czech Republic	(-)
C102/13C	2013	Mnichovo Hradiště - Středočeský kraj	Czech Republic	(-)
C5/12C	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C6/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C11/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C19/12A	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)

Table 1. (Continued)

Code of samples	Year of collection	Locality	Country	dsRNA bands
C09/12A	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C99/13B	2013	Vranovice	Czech Republic	(-)
C28/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C60/12C	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C66/12A	2012	Krásné Pole - Ústecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
C40/12B	2012	Ploužnice´- Liberecký kraj	Czech Republic	2,5kb
C13/12B	2012	Výrava - Královéhradecký kraj	Czech Republic	(-)
C50/12B	2012	Lovečkovice -Ústecký kraj	Czech Republic	4.5kb
C74/12C	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C107/13B	2013	Teplice - Ústecký kraj	Czech Republic	(-)
C48/12B	2012	Krásné Pole - Ústecký kraj	Czech Republic	(-)
C103/13B	2012	Krásné Pole - Ústecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
C5/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C61/12C	2012	Ploužnice - Liberecký kraj	Czech Republic	(-)
C34/12B	2012	Ploužnice - Liberecký kraj	Czech Republic	2.2kb, 2.5kb, 4.5kb
GRO 2	2010	Gröbming, Styria	Austria	(-)
KAR 4	2009	Karlsbach, Upper Austria	Austria	2.5kb, 4.5kb
HAS/FP/4/2	2011	Hasenauerstrasse, Vienna	Austria	(-)
GB 14	2011	Gansbach, Lower Austria	Austria	(-)
HO/II/6/1	2008	Hohenau an der March, Lower Austria	Austria	2.2kb, 2.5kb, 4.5kb
KAB 9	2009	Kapuzinerberg, Salzburg	Austria	(-)
BI/2/2	2009	Bad Ischl, Upper Austria	Austria	(-)
BB/1/19	2009	Bisamberg, Lower Austria	Austria	(-)
VER/2	2009	Verditz, Carinthia	Austria	(-)
N5/4/A	2007	Altaussee, Styria	Austria	2.2kb, 2.5kb
LOF 5	2009	Lofer, Salzburg	Austria	(-)
SAB 2/1	2008	Saberda, Carinthia	Austria	(-)
KOR/FP/17/2	2011	Langenzersdorf, Lower Austria	Austria	2.2kb, 2.5kb, 4.5kb
STL 1/6	2010	Lorenzen ob Murau, Styria	Austria	(-)
KOR/FP/7	2011	Langenzersdorf, Lower Austria	Austria	(-)
KOR/FP/3	2011	Langenzersdorf, Lower Austria	Austria	2.5 kb
BIZ/1	2010	Bizau, Vorarlberg	Austria	(-)
GOT 1/1/2	2009	Götzis, Vorarlberg	Austria	(-)
T 4/2	2013	Kolsass, Tyrol	Austria	(-)
WER 2/17	2009	Werfen, Salzburg	Austria	(-)
TU 3/2/1/1	2008	Tulln, Lower Austria	Austria	2.2kb, 2.5kb
KOR/FP/7	2011	Langenzersdorf, Lower Austria	Austria	(-)
SFB/II/7/2	2008	Vienna-Schafberg, Vienna	Austria	(-)
ST1	2010	Stinaz, Burgenland	Austria	(-)
NWE/1/2/H1	2008	Vienna-Neuwaldegg, Vienna	Austria	(-)
N 1/3/Holz	2008	Vienna-Neuwaldegg, Vienna	Austria	(-)
Z E7/48	2014	Zbojská	Slovakia	(-)
Z E17/5 1	2014	Zbojská	Slovakia	4.5kb
Z E7/48 1	2014	Zbojská	Slovakia	(-)
Z O 13/10	2014	Zbojská	Slovakia	(-)
Z G13/10 2	2014	Zbojská	Slovakia	(-)
Z B17/44	2014	Zbojská	Slovakia	(-)
Z J15/55 2	2014	Zbojská	Slovakia	(-)
Z E13/42 4	2014	Zbojská	Slovakia	(-)
Ú 3o	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 1T	2014	Úľany nad Žitavou	Slovakia	2.2 kb
Ú 1g	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 1B	2014	Úľany nad Žitavou	Slovakia	(-)
Ú 3i	2014	Úľany nad Žitavou	Slovakia	(-)

Table 1. (Continued)

Code of samples	Year of collection	Locality	Country	dsRNA bands
Ú1k	2014	Úľany nad Žitavou	Slovakia	(-)
Č 2J	2014	Černík	Slovakia	2.5 kb
Č 1c	2014	Černík	Slovakia	(-)
Č 2H	2014	Černík	Slovakia	2.2kb, 2.5kb
Č 2S	2014	Černík	Slovakia	(-)
Č 2Q	2014	Černík	Slovakia	(-)
Č 2G	2014	Černík	Slovakia	(-)
Č 2x	2014	Černík	Slovakia	2.5 kb
TR 19	2014	Trstice	Slovakia	(-)
Hladomer 4	2013	Lovce	Slovakia	(-)
Hladomer 1	2013	Lovce	Slovakia	(-)
Príbelce 3	2013	Príbelce	Slovakia	(-)
Štitáre 6E	2013	Štitáre	Slovakia	(-)
Štitáre 5B	2013	Štitáre	Slovakia	(-)
Jarok 2	2013	Jarok	Slovakia	(-)
Jarok 1a4	2013	Jarok	Slovakia	4.5kb
L9	2014	Ladzany	Slovakia	(-)
SA PC 2 E	2013	Svätý Anton	Slovakia	(-)
SA NI 7B	2013	Svätý Anton	Slovakia	(-)
SA 8 A	2013	Svätý Anton	Slovakia	(-)
SA 2/B	2013	Svätý Anton	Slovakia	(-)
SA NC 2A	2013	Svätý Anton	Slovakia	(-)
SA PC 2A	2013	Svätý Anton	Slovakia	2.2 kb
SA NC 1C	2013	Svätý Anton	Slovakia	(-)
SA NI 6B	2013	Svätý Anton	Slovakia	(-)
SA NI 10C	2013	Svätý Anton	Slovakia	(-)

(KJ667051). Unexpectedly, in contrast with the Blastn result of *Sclerotinia sclerotiorum* mitovirus 5, mapping/aligning was not successful and no coverage was determined (KJ62509) (Khalifa and Pearson, 2014).

Genome organisation of HfMV1-strain 1739

The total length of this dsRNA segment was 2350 nt with a 44.5 % GC content. Using the mitochondrial translation table, a single large ORF of 2154 nt was determined. The putative start codon AUG, occurring in an AU-rich context, was located at position 197, and the stop codon UAG was located at position 2348, potentially yielding a protein of 783 aa in length with a predicted molecular weight of 87.308 KDa, an isoelectric point of 10.13 and an extinction coefficient of 137,905.

A comparison of the full-length genome sequence in GenBank (Blastx) revealed the highest similarity with HfMV1 (88 %), followed by *Cryphonectria cubensis* mitovirus 1a (51 %) and *Sclerotinia sclerotiorum* mitovirus 15 (50 %). In addition, the alignment of the identified sequences with the Polish and German strains of HfMV1 (Schoebel et al. 2014) revealed 2,202 (93.7%) identical sites, and an overall pairwise identity of 95.4 %. According to the nomenclature practices of Schoebel *et al.* (2014), this strain was designated H. fraxineus mitovirus 1-strain 1739.

Discussion

Sequencing is the most reliable and adequate method for identifying fungal viruses, but the molecular weight of the dsRNA banding patterns might indicate the type of putative viruses involved and facilitate further analyses (Herrero et al. 2009; Botella et al. 2011). In the present study, we screened potential virus molecules in 134 isolates of *H. fraxineus* obtained from three different countries in Central Europe. Three different dsRNA bands of *ca* 2.2, 2.5 kb and 4.5 kb were confirmed to occur at a moderate frequency (28.4 % of the total contained the three bands). Different factors might influence the dsRNA frequency within fungi, i.e., the type of spore (sexual vs. asexual) (Pearson et al. 2009) that the fungus uses as its primary reproduction system. Conidiospores are more efficient for transferring mycoviruses, while virus transmission through sexual spores is a less efficient particularly in ascomycetes (Polashock et al. 1997; Buck, 1998; Pearson et al. 2009). Ascospores are the primary dispersion and infectious mechanism of *H. fraxineus* in nature (Gross et al. 2014b), and recently it was also shown that HfMV1 is vertically transmitted into sexual ascospores at a high frequency (Schoebel et al. 2017). However, in the present study, only mycelia derived from lesions were analysed. As lesions are consid-

ered a dead-end for the fungus, this might potentially decrease the capacity of mycovirus transfer. On the other hand no significant differences in prevalence, genotype diversity and virulence could be detected for HfMV1 (Burokiene et al. 2015, Lygis et al. 2016, Schoebel et al. 2017). The observed rates of virus transmission greatly differ for diverse fungus/virus combinations (Pearson et al. 2009). The transmission of mycoviruses is also controlled through a genetic self/non self-recognition system designated heterokaryon or vegetative incompatibility (vic). Although little is known about the vegetative compatibility groups (VCGs) of *H. fraxineus* in Europe, Brasier and Webber (2013) showed that in the UK, most genetic *H. fraxineus* individuals are likely vegetatively incompatible, suggesting that vegetative incompatibility indirectly influences the flow of viruses in *H. fraxineus*.

The first two bands observed were approximately 2.2 and 2.5 kb in size, which could be preliminarily classified as members of the family *Narnaviridae,* such as the genera *Mitovirus* or *Narnavirus*. Based to Schoebel *et al.* (2014), which previously described the full-length sequence of Hymenoscyphus fraxineus mitovirus 1 (HfMV1) in one Polish and one German *H. fraxineus* isolate, we used RT-PCR and specific-primers to determine whether HfMV1 corresponds to the *ca* 2.2 and/or 2.5 kb bands. Moreover, HfMV1 was identified in positive *H. fraxineus* isolates examined. In parallel, the direct high-throughput sequencing of one Czech isolate hosting the three determined bands (Figure 1) corroborated the presence of HfMV1 and also confirmed the presence of this virus in Austria, Czechia and Slovakia, as it has been recently published by Schoebel *et al.* (2017).

The novel defined Czech strain (sequence) was called HfMV1-strain 1739 and according to its size (2350 nt), this virus could correspond to a band of approximately 2.5 kb. This sequence was slightly shorter than the Polish sequence C402 (2439 nt) and slightly longer than the German sequence C9444 (2281 nt) (Schoebel et al. 2014). HfMV1-1739 is the only mitovirus species detected in the pool of data, and the fact that we detected HfMV1 in isolates harbouring both or only one band suggested that both bands might correspond to HfMV1. We confirmed this supposition through the direct RT-PCR of the cDNA from each band. However, whether the smaller segment (2.2 nt) has a shorter ORF or whether part of the 5' and/or 3' untranslated regions (UTRs) is lost should be further addressed. Mitoviruses are widespread in pathogenic fungi, such as *Ophiostoma novo-ulmi* (Cole et al. 1998), *Gremmeniella abietina* (Tuomivirta and Hantula, 2003; Botella et al. 2012), *Rhizoctonia cerealis* (Zhang et al. 2015), *Fusarium coeruleum*, *Fusarium globosum* (Osaki et al. 2015), *Fusarium circinatum* (Martinez-Alvarez et al. 2014), *Thielaviopsis basicola* (Park et al. 2006), *Helicobasidium mompa* (Osaki et al. 2005) and *Heterobasidion annosum* (4.4 kb) (Vainio et al. 2015). However, no evidence of the potential loss of part of the genome has been ever reported in mitoviruses.

The third band was 4.5 kb in size and remains unidentified; thus, further analyses are required. Nevertheless, we hypothesize this band does not belong to HfMV1, as the direct specific RT-PCR of the corresponding cDNA was negative in the isolate MeU_1739. According to the molecular weight of the detected segment (4.5 kb), this virus could be included in the family *Totiviridae*, which normally contains a monosegmented dsRNA of 4.6-7.0 kb. Totiviruses have been reported in both *Diplodia pinea* and *D. Scrobiculata* (Wet et al. 1998) and *Gremmeniella abietina* (Tuomivirta and Hantula 2005). However, we did not detect any putative member of *Totiviridae* among the pool of identified sequences. Therefore, although we cannot fully discard this idea, this virus might belong to another viral family or even a novel species belonging to an as yet undescribed virus family, not included in the reference libraries. Moreover, the intensity of this dsRNA segment is markedly faint (Fig. 1), suggesting that 4.5 kb dsRNA might correspond to a virus with ssRNA genome and low copy numbers of replicative dsRNA (Vainio et al. 2015).

In conclusion, the results of the present study confirm the presence of three dsRNA bands within *H. fraxineus* in Austria, Czech Republic and Slovakia. Two of them (2.2 and 2.5 kb) belong to HfMV1 and the third (4.5 kb) suggests the occurrence of another putative novel virus, which remains unidentified and suggests that there is another mycovirus present in *H. fraxineus*. Furthermore, a novel full-length strain of HfMV1 is described. This work contributes to an ongoing attempt to understand the role of mycoviruses as potential biological control agents (BCAs) for *H. fraxineus*.

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