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Short communication

Mitoviruses in the conifer root rot pathogens *Heterobasidion annosum* and *H. parviporum*

Eeva J. Vainio

Natural Resources Institute Finland, Latokartanonkaari 9, 00790 Helsinki, Finland

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ABSTRACT

Mitoviral infections are highly common among fungi, but so far only one mitovirus has been described in *Heterobasidion* spp. conifer pathogens. Here, the occurrence of further mitoviruses was investigated using a previously published RNA-Seq dataset for *de novo* contig assembly. This allowed the identification of two additional mitovirus strains designated as Heterobasidion mitovirus 2 (HetMV2) and HetMV3 with genome lengths of ca. 2.9 and 5.0 kb. Furthermore, the occurrence of similar viruses was screened among a collection of *Heterobasidion* isolates using RT-PCR. Mitoviruses were detected in six more fungal isolates and two different host species, *H. annosum* and *H. parviporum*.

The virus family *Narnaviridae* encompasses viruses infecting yeasts (genus *Narnavirus*) or filamentous fungi (genus *Mitovirus*). These mycoviruses are non-encapsidated and have small positive-sense ssRNA genomes encoding only a single protein, the RNA dependent RNA polymerase (RdRP) (Hillman and Esteban, 2011). Members of genus *Narnavirus* replicate in the host cytosol, whereas members of genus *Mitovirus* reside inside host mitochondria, and their RdRP is typically encoded using the mitochondrial translation table, where the codon UGA encodes for tryptophan instead of a stop codon. There are five classified species of mitoviruses, but many more strains with complete published genome sequences await formal classification (Hillman and Cai, 2013; Marais et al., 2017). Recently, mitoviruses have been shown to infect also plants (Nibert et al., 2018; Nerva et al., 2019). Based on phylogenetic analysis, the polymerase sequences of mitoviruses resemble those of the newly established family *Botourmiaviridae* (Taxonomy proposal 2018.003 F.A.v1; https://talk.ictvonline.org) and RNA bacteriophages of the family *Leviviridae* (Koonin and Dolja, 2014; Lefkowitz et al., 2018; Wolf et al., 2018).

The fungal genus *Heterobasidion* includes some of the most destructive conifer pathogens of the northern Boreal forest region (Garbelotto and Gonthier, 2013). These fungi form large disease centers by vegetative spread via tree root contacts, and produce basidiospores that infect freshly cut conifer stumps or wounded trees. *Heterobasidion* species host diverse members of family *Partitiviridae* (Vainio and Hantula, 2016), and ubiquitous infections of an unclassified virus species called Heterobasidion RNA virus 6 (HetRV6), which is related to the mutualistic Curvularia Thermal Tolerance virus (Márquez et al., 2007).

The existence of mitoviruses in *Heterobasidion annosum* was observed earlier using deep sequencing of small RNAs (Vainio et al., 2015a) and RNA-Seq (Vainio et al., 2018). During those studies, various mitoviral sequence fragments were detected in *H. annosum* strain 94233 that also harbors Heterobasidion partitivirus 13 strain an1 (HetPV13-an1) causing strong growth debilitation in its host (Vainio et al., 2018). The same host strain containing only mitoviral infections displays normal morphology and growth (Vainio et al., 2018). The complete genome sequence of one mitovirus from this host has been characterized earlier (Vainio et al., 2015a). Here, the complete genome sequences of two co-infecting mitovirus strains are described, and the occurrence of similar virus strains among other *Heterobasidion* isolates is reported.

Using cs. 117 M RNA-Seq reads obtained from three biological replicate samples of *H. annosum* strain 94233 (Vainio et al., 2018), nearly complete genome sequences of the three different mitovirus strains could be deduced. First, all sequence reads were mapped against the *H. irregulare* genome sequence available at the JGI (http://genome.jgi.doe.gov/Hetan2/Hetan2.home.html). Unaligned reads were then *de novo* assembled with Geneious R10 (Biomatters Ltd., New Zealand) Tadpole assembler (k-mer = 69; minimum contig length = 201), and the resulting contigs were mapped again against the host genome. The unmapped contigs were examined for sequence homology against the GenBank viruses database (taxid 10239) using NCBI BlastX and BlastN with expect threshold of 0.1. Contigs with mitovirus affinities were used for contig extension steps with Geneious R10 based on iterative mapping until no further reads mapped to the extended contig. Table 1 presents the mapping statistics based on RNA-Seq reads from *H.*

E-mail address: eeva.vainio@luke.fi.

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Table 1
Mapping of small RNA and RNA-Seq reads to Heterobasidion mitovirus sequences.

	Reference sequence length (nt)	Coverage[a] small RNAs	Depth[b] small RNAs (mean)	N° mapped small RNA reads[c]	Read length small RNAs	Coverage RNA-Seq[d]	Depth[b] RNA-Seq (mean)	N° mapped reads RNA-Seq	Read length RNA-Seq
HetMV1-an1	4379	98.8	147	28962	18-27	99.4	63.6	2766	101
HetMV2-an1	2923	98.7	53	6979	18-27	99.5	31.5	916	101
HetMV3-an1	4955	99.5	85	19002	18-27	99.3	59.2	2918	101

[a] Sequence coverage is the percentage of the full reference sequence covered by reads.
[b] Depth is the average number of reads per sequence position.
[c] Mapping conducted with Geneious R10 Bowtie short read mapper (max two mismatches; seed length of 11; best match only). The data included 11,974,256 reads that remained unassembled against the host genome. Note that there may be some misassembly between HetMV1-an1 and HetMV3-an1 due to high sequence similarity and shortness of the small RNA reads.
[d] Mapping conducted with Geneious R10 (low sensitivity). The data included three independent biological replicates with a total of 116,891,112 reads (Vainio et al., 2018). The reads were uniquely mapped to HetMV1-an1, HetMV2-an1 and HetMV3-an1.

annosum 94233 (deposited in GenBank SRA database under accession number SRP097618 (Bioproject accession number PRJNA362289; Vainio et al., 2018), as well as small RNA reads derived from a pool of *Heterobasidion* strains (Vainio et al., 2015a).

One of the mitoviral contigs represented the previously published sequence of Heterobasidion mitovirus 1 (HetMV1) (Vainio et al., 2015a). The entire sequences of two other mitoviral contigs were verified by Sanger sequencing of PCR amplicons, using at least two independent cDNA samples for each isolate. Sequence ends were determined by ligating an adapter to the 3´ ends of the replicative dsRNA intermediates to provide a priming site for cDNA synthesis and subsequent PCR amplification as described earlier (Lambden et al., 1992; Vainio et al., 2011a). Sanger sequencing was conducted at Macrogen Europe (www.macrogen.com).

The complete genome sequence of the first new mitovirus described here and designated as Heterobasidion mitovirus 2 strain 1 from *H. annosum* (HetMV2-an1) comprises 2923 nt (Fig. 1). The genome includes a conserved region resembling the Mitovirus RdRP subfamily pfam05919 (aa residues 197-608; E-value 1.03e-86). It has one large ORF predicted to encode an RdRP of 756 aa using translation table 4 (nt sites 275-2542; predicted M_r = 86.167 kDa). This ORF is interrupted by six UGA stop codons if translated using the standard nuclear translation table (Fig. 1). The G + C-contents of the ORF region and full genome sequence are 37.8% and 39.1%, respectively. Based on the RNA-Seq reads and RT-PCR amplification using specific primers, the 3´-terminus of the virus genome comprises a highly GC-rich sequence of 17 nucleotides which may contain length polymorphisms as observed earlier for Gremmeniella mitovirus S1 (Tuomivirta and Hantula, 2003).

The second new mitovirus detected in *H. annosum* 94233 has one long ORF predicted to encode a putative RdRP of 816 aa (nt sites 717-3164; predicted M_r = 92.001 kDa) when translated using mitochondrial codon usage (Fig. 1). It was provisionally named HetMV3-an1 (Heterobasidion mitovirus 3 strain 1 from *H. annosum*). The G + C-contents are 42.8% and 43.4% for the RdRP encoding ORF region and full sequence, respectively. The predicted RdRP sequence shares ca. 68% aa identity with the previously described strain HetMV1-an1. Notably, HetMV1-an1 and HetMV3-an1 have identical sequences at their terminal ends. Thus, the first eleven 5´-terminal nucleotides (GGGGTAGA CTC) as well as the last eleven 3´–terminal nucleotides (GGCAGGAC CCC) are identical between the two virus strains. The first and last five nucleotides are also palindromic, a typical feature appearing in many other mitoviruses, such as the classified Ophiostoma mitovirus 4 and Ophiostoma mitovirus 6 (Hillman and Esteban, 2011). Predicted panhandle structures resulting from terminal inverse complementarity are variably found among mitoviruses (e.g., Hong et al., 1998a, 1999; Lakshman et al., 1998; Osaki et al., 2005). Notably, HetMV1-an1 and HetMV3-an1 differ considerably in the size of their 3´ UTR region, which is 1068 nt in HetMV1-an1 and 1788 nt in HetMV3-an1. Consequently, HetMV3-an1 genome comprises 4955 nt, whereas HetMV1-an1 includes 4379 nt. Additional short ORFs not present in HetMV1-an1 were found in the 3´ UTR of HetMV3-an1, but none of these gave significant hits using BlastP or tBlastX of the NCBI database (data not shown). Sanger sequencing of two independent cDNA samples different from the one subjected to RNA-Seq was used to confirm the sequences. Classified mitoviruses have genomes of ca. 2.3–2.7 kb with relatively short 3´ UTR regions (43–212 nt) (Hillman and Esteban, 2011). However, large 3´ UTR sequences of 749–952 nt have been found in the unclassified mitoviruses Tuber aestivum mitovirus (Stielow et al., 2011), Botrytis cinerea debilitation-related virus (Wu et al., 2010), and Thanatephorus cucumeris mitovirus (Lakshman et al., 1998). Overall, there seems to be large variation in the length of the nontranslated sequences in mitoviruses (Stielow et al., 2011; Xie and Ghabrial, 2012). Apart from a few mitoviral sequences of up to 5.2 kb reported in a soil metatranscriptomics dataset (Starr et al., 2019; GenBank accession MN033892), the genome sequence of HetMV3-an1 seems to be the longest one among publicly available mitoviral sequences (in total 2236 sequences in the NCBI GenBank nucleotide database found using search term “Mitovirus” [Organism]; accessed 31 July 2019).

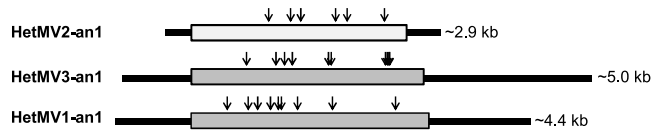

Fig. 1. Genome organization of Heterobasidion mitoviruses in *H. annosum* 94233. Untranslated regions are shown in black, and arrows indicate the positions of UGA codons encoding tryptophan within predicted RNA-dependent RNA polymerase encoding regions when translated using the mold mitochondrial codon usage (translation table 4).

No mitoviral amplification products were detected using total DNA of the host *H. annosum* 94233 as a PCR template with specific primers, and therefore the new mitovirus strains did not appear to be integrated into the host genome (Liu et al., 2010; Chiba et al., 2011). The following specific primer pairs were used: MV346 F (5´-CAGGAATGGAT AATAGCTTCG-3´) and 3rdMVMidR1 (5´-AACATTTCCGCATCCGTTAG-3´) for HetMV3-an1; 2ndMVEndF3 (5´-GATCAAACCACACGGGACTT-3´) and 2ndMVEndRev (5´-CGTCTCCGGCATTACAATTT-3´) for HetMV2-an1. These primers produce amplicons of 409 nt and 524 nt from HetMV2-an1 and HetMV3-an1, respectively. Reverse-transcribed total RNA of the same host isolate was used as a positive control for viral amplification, and the quality of the DNA samples was verified by M13 minisatellite fingerprinting as described earlier (Stenlid et al., 1994; Vainio et al., 2012). It should also be noted that the mitoviral sequences detected in *H. annosum* 94233 did not share significant sequence similarity with the mitochondrial plasmids integrated in the mitochondrial genome of *H. irregulare* strain TC 32-1 by Himmelstrand et al. (2014).

Species demarcation criteria for mitoviruses have not been precisely defined (Hillman and Esteban, 2011). Among the classified mitoviruses, isolates of different species have been reported to share less than 40%

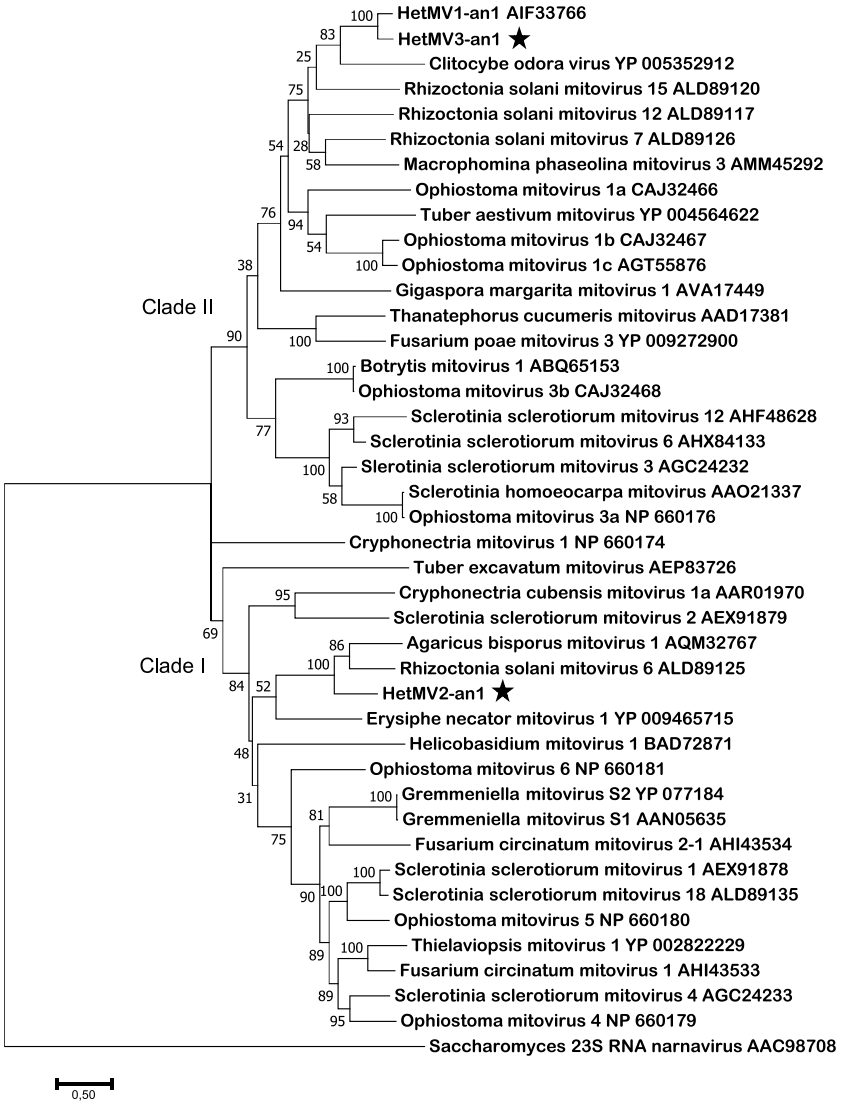

Fig. 2. Phylogenetic analysis of predicted RdRP aa sequences of selected mitoviruses. The analysis was conducted using Maximum Likelihood method based on the Le Gascuel 2008 model (LG + *G* + *I*). Bootstrap support based on 100 repetitions is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The aa sequence alignments were generated by MAFFT and evolutionary analyses were conducted in MEGA7 (http://www.megasoftware.net/).

aa sequence identities of putative RdRP proteins, whereas isolates of the same species have shown higher than 90% sequence identities. Among the mitovirus strains described here, HetMV2 seems to constitute a new species of genus *Mitovirus*. Based on global alignment with MAFFT, it shares approximately 23% RdRP aa sequence identity with the co-occurring virus strains HetMV1-an1 and HetMV3-an1, and ca. 44% RdRP aa identity (query cover 87%; E-value 6e-160) with the most similar virus strain, Rhizoctonia solani mitovirus 6 (Marzano et al., 2016). Phylogenetic analysis of mitoviral RdRP sequences (Fig. 2), indicated that HetMV2 clustered within the "Clade I" of genus *Mitovirus* (Hillman and Cai, 2013). It formed a highly supported subcluster with Rhizoctonia solani mitovirus 6 and Agaricus bisporus mitovirus 1, both of them found in a basidiomycete host.

Phylogenetic analysis showed that HetMV1-an1 and HetMV3-an1 clustered within the "Clade II" of genus *Mitovirus* (Hillman and Cai, 2013), and formed a subgroup with the Clitocybe odora mitovirus (Heinze, 2012). The presence of identical terminal sequences and relatively high RdRP aa sequence identity (68%) suggests that HetMV1-an1 and HetMV3-an1 might be considered conspecific. In that case, the mixed infection would contradict a species demarcation criterion for the related genus *Narnavirus* stating incompatibility of conspecific narnaviruses within the same host strain. In the case of mitoviruses, the existence of several virus strains within single host isolates seems to be a common phenomenon (e.g., Hong et al., 1999; Osaki et al., 2016), but in most cases the co-infecting mitoviruses are distantly related (Hillman and Cai, 2013). Notably, based on transmission frequencies of mitoviruses to ascospore progeny in *Cryphonectria parasitica*, Polashock et al. (1997) suggested that only a subset of mitochondria may be virus infected. Correspondingly, the occurrence of multiple mitochondria in single host cells might enable the co-existence of several similar mitovirus strains in a single mycelium (in contrast to cytosolic narnaviruses).

The occurrence of large size differences in the nontranslated regions of the similar HetMV1 and HetMV3 strains suggests that *Heterobasidion* mitoviruses may be prone to recombination. To investigate this possibility, the nucleotide sequences of the three co-infecting strains were subjected to recombination detection analysis by the RDP program RDP4 (v.4.16; Martin et al., 2010). Interestingly, a recombination event was detected in the 3´ UTR shortly outside the ORF region (130 and 140 nt downstream from the stop codons of HetMV2-an1 and HetMV1-an1, respectively). In that sequence region comprising ca. 70 nucleotides, HetMV3-an1 and HetMV2-an1 shared over 90% sequence

Table 2
Heterobasidion isolates with mitovirus infections as determined by RT-PCR with selective primers and mitovirus sequence information.

Isolate	Species	Collection site	Mitovirus infection (s)	GenBank accession numbers	Sequence length (nt)	Other known viruses in the host	Study describing the host
94233	*H. annosum*	Podanin, Poland	HetMV1-an1, HetMV2-an1, HetMV3-an1	KJ873059, MN058206, MN058208	4379, 2923, 4955	HetPV13	Vainio et al., 2015a
94245	*H. annosum*	Podanin, Poland	HetMV2-an2 HetMV3-an2,	MN058207, MN058209	2418[b] 3229[b]	HetRV6, HetPV11	Vainio et al., 2012
82-12[a]	*H. parviporum*	Tuusula, Finland	HetMV3-pa1-1	MN058210	3241[b]	HetRV6	Vainio et al., 2015b
124-12[a]	*H. parviporum*	Tuusula, Finland	HetMV3-pa1-2	MN058211	2728[b]	HetRV6	Vainio et al., 2015b
64-12	*H. parviporum*	Tuusula, Finland	HetMV3-pa-1-3	MN058212	755[b,c]	None	Vainio et al., 2015b
14/C	*H. parviporum*	Ruotsinkylä, Finland	HetMV3-pa2	MN058213	3204[b]	HetRV6, HetPV4	Vainio et al., 2013
95162	*H. parviporum*	Perm, Russia	HetMV3-pa3	MN058214	3580[b]	HetRV6	Vainio et al., 2012

[a] Also the following isolates representing the same clonally spreading *Heterobasidion* strain were infected by HetMV3 as determined by RT-PCR with selective primers: 82-05, 83-12, 123-12, 123-05, 124-12, 124-05.
[b] The sequence is partial.
[c] The sequence was determined in one direction only.

identity, whereas their sequence identities compared to HetMV1-an1 were only 51–55%.

The presence of three co-infecting mitoviruses in a single isolate of *H. annosum* also suggested that mitoviruses might be common in strains of *Heterobasidion* although none had been detected by conventional virus screening based on the presence of dsRNA. Hence, primers based on the mitoviral sequences were used to screen for the presence of similar virus infections in collections of *Heterobasidion* fungi (Table 2, Table S1). The initial screening was conducted using the following selective primer pairs: 3rdCONFor1 (5´-AAGCTGCGGGTAAAGTGAGA-3´) & 3rdCONRev1 (5´-CTATAGTCCCGTCCGACCAA-3´), and 2ndCONF1 (5´-CGCTATCTTCGATTACTGATCACA-3´) & 2ndCONRev2 (5´-GCTATACGCTCCCATTGGTTG-3´). The former primer pair matches perfectly the genome sequence of HetMV1-an1, but produces amplicons of 298 bp also from strains of HetMV3 (each primer has 0–2 mismatches to known HetMV3 sequences), whereas the second primer pair is specific to HetMV2 and produces an amplicon of 349 bp.

The screening of a collection of 52 *Heterobasidion* spp. samples originating from previous population studies (Vainio et al., 2012, 2013, 2015b; Hyder et al., 2018; Table 2, Table S1) resulted in the discovery of one additional HetMV2 strain, and four variants of HetMV3 (no additional HetMV1 strains were found). The phenotypes of the *Heterobasidion* strains infected with mitoviruses were not investigated in detail during this study, but they did not seem to exhibit noticeable symptoms of debilitation. Interestingly, most of the mitoviruses detected in this study occurred in mixed infections with Heterobasidion partitiviruses and the unclassified Heterobasidion RNA virus 6 (see Table 2). Mitoviruses were detected by RT-PCR in two host species, *H. annosum* and *H. parviporum*, which comprised most of the isolates included in the analysis (Table 2, Table S1). Their dsRNA replicative intermediates had not been detected during earlier investigations using cellulose affinity chromatography (e.g., Vainio et al., 2011b; Kashif et al., 2015).The RdRP-encoding genome region of the newly detected viruses was targeted for sequencing with selective primers using PCR amplicons generated with PHUSION high fidelity DNA Polymerase and/or DynaZyme II DNA Polymerase (Thermo Fischer Scientific).

The *H. parviporum* strains from Southern Finland (Ruotsinkylä, Tuusula) had been collected from two forest sites located approximately 1.6 km apart. HetMV3 strains from these sites (HetMV3-pa1 with three variants and HetMV3-pa2; see Table 2) shared ca. 93% global nucleotide sequence identity over ca. 3.2 kb including the full ORF region. Approximately the same level of sequence identity was observed between these Finnish virus strains and another HetMV3 strain originating from a Russian *H. parviporum* strain from Ust-Kisert in Perm, which is located ca. 1860 km apart from the Finnish forest sites (Table 2). Virus variants from one Finnish collection site in Tuusula from the same or different clonal host individuals shared over 99% nt sequence identity. For example, nearly identical HetMV3 variants were found in isolates 82-12 and 64-12 that represented two different *H. parviporum* clones at the site (Table 2). The *H. parviporum* isolate 82-12 was part of a large clonal individual that had infected several trees and stumps, and the other isolates were also investigated for the presence of HetMV3. In this case, the virus had spread to most parts of the clonal *H. parviporum* mycelium, and there seemed to be minor sequence variations among virus isolates from *Heterobasidion* subcultures isolated from different trees or stumps. This resembles the polymorphisms observed earlier in partitiviruses and HetRV6 strains at the same forest site (Vainio et al., 2015b). After detecting HetMV3, the presence of the virus was investigated also among isolates collected from the same site by basidiospore trapping (N = 50; Vainio et al., 2015b), but no mitovirus infections were detected in the spore-derived isolates (data not shown).

The two HetMV3 strains from *H. annosum* isolates 94233 and 94245 collected ca. 38 km distance apart in Poland (Krucz and Podanin) were about 98% identical in sequence over ca. 3.2 kb (Table 2), whereas the HetMV2 strains in these same two isolates shared ca. 94% nt sequence identity over ca. 2.4 kb. On the other hand, HetMV3 strains from the two different host species, *H. annosum* and *H. parviporum,* shared only ca. 89% nt sequence identity (notably, there was also a considerable distance between the collection sites of these isolates: ca. 956–983 km between the Finnish and Polish forest sites, and ca. 2560 km between the Russian and Polish sites).

In conclusion, this investigation shows that mitoviruses are relatively common among isolates of *Heterobasidion* species. As typical for many mitoviruses, single host isolates were shown to harbor mixed virus infections. Future high-throughput sequencing studies are expected to further enlighten the diversity of mitoviruses infecting these forest pathogenic fungi.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197681.

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