



## Population structure of a novel putative mycovirus infecting the conifer root-rot fungus *Heterobasidion annosum* sensu lato

Eeva J. Vainio<sup>a,\*</sup>, Rafiqul Hyder<sup>a</sup>, Gülden Aday<sup>b</sup>, Everett Hansen<sup>c</sup>, Tuula Piri<sup>a</sup>, Tuğba Doğmuş-Lehtijärvi<sup>b</sup>, Asko Lehtijärvi<sup>b</sup>, Kari Korhonen<sup>a</sup>, Jarkko Hantula<sup>a</sup>

<sup>a</sup> Finnish Forest Research Institute, Jokiniemenkuja 1, P.O. Box 18, 01301 Vantaa, Finland

<sup>b</sup> Süleyman Demirel University, Faculty of Forestry, Department of Botany, 32260 Isparta, Turkey

<sup>c</sup> Oregon State University, Department of Botany and Plant Pathology, 2082 Cordley Hall, Corvallis, OR 97331, USA

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### ABSTRACT

We describe a novel putative mycovirus infecting the conifer root-rot fungus *Heterobasidion annosum* sensu lato. This virus, designated as *Heterobasidion* RNA virus 6 (HetRV6), is taxonomically distant from all previously known viruses of *Heterobasidion* species, but somewhat related to the *Curvularia* thermal tolerance virus and the *Fusarium graminearum* virus 4. Based on a population analysis including 35 virus strains from *Heterobasidion abietinum*, *Heterobasidion parviporum*, *Heterobasidion annosum* sensu stricto and *Heterobasidion occidentale*, HetRV6 showed a considerable degree of geographical and host-related differentiation. The North American and Eurasian virus populations were clearly separated. In Eurasia, we observed cases of discrepancy between virus and host taxonomy, suggesting interspecies virus transfer. HetRV6 was also successfully transmitted between the three European species *H. abietinum*, *H. annosum* and *H. parviporum*. Based on growth rate tests on agar plates and spruce stem pieces, HetRV6 seemed to be cryptic or slightly mutualistic to its host.

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### Introduction

Viruses occur commonly in all major groups of the true fungi (Ghabrial and Suzuki, 2009; Pearson et al., 2009). Fungal viruses (mycoviruses) have genomes composed of single-stranded (ss) or double-stranded (ds) RNA, or rarely DNA (Yu et al., 2010). Currently, mycoviruses are classified according to their genomic composition into four dsRNA virus families (*Chrysoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*) and five ssRNA virus families (*Barnaviridae*, *Hypoviridae*, *Narnaviridae*, *Pseudoviridae* and *Metaviridae*; International Committee on Taxonomy of Viruses, ICTV; [www.ictvonline.org](http://www.ictvonline.org)).

During recent years, several novel mycovirus taxa have been described. Some of these new virus species, like the *Curvularia* thermal tolerance virus, the *Rosellinia necatrix* megabirnavirus 1, and the *Gremmeniella abietina* type B RNA virus XL, are distant from all previously known mycoviruses (Chiba et al., 2009; Márquez et al., 2007; Tuomivirta et al., 2009). Many also resemble plant viruses: the *Botrytis* virus X, the *Fusarium graminearum* virus DK21 and the *Sclerotinia sclerotiorum*

debilitation-associated RNA virus resemble positive-strand RNA plant viruses in the family *Flexiviridae* (Howitt et al., 2006; Kwon et al., 2007; Xie et al., 2006), while the *Diaporthe ambigua* RNA virus has a distant relationship to the plant virus family *Tombusviridae* (Preisig et al., 2000). On the other hand, the reovirus *Cryphonectria parasitica* 9B21 and the *S. sclerotiorum* RNA virus L are related to animal pathogenic viruses (Hillman et al., 2004; Liu et al., 2009).

Most mycoviruses are cryptic and seem to have no phenotypic effects on their host fungi. However, some are detrimental to their host and can mediate hypovirulence (reduction of virulence) in plant pathogenic fungal species. This phenomenon was originally described for hypoviruses of *C. parasitica*, and many other examples have since been found (Anagnostakis and Day, 1979; Deng et al., 2003; Huang and Ghabrial, 1996; Lakshman et al., 1998; Preisig et al., 2000; Yu et al., 2010). Recently, also mutualistic associations have been described: the *Nectria radicola* virus L1 enhances the virulence of its plant pathogenic host fungus (Ahn and Lee, 2001), and the *Curvularia* thermal tolerance virus is involved in a three-way symbiosis with an endosymbiont fungus and its host grass (Márquez et al., 2007).

The fungal genus *Heterobasidion* consists of two species complexes: *Heterobasidion annosum* (Fr.) Bref. sensu lato and *Heterobasidion insulare* (Murrill) Ryvarden sensu lato. The *H. insulare* complex includes mostly saprotrophic wood-decay fungi that occur in eastern Asia (Dai

\* Corresponding author. Fax: +358 10 211 2206.  
E-mail address: [eva.vainio@metla.fi](mailto:eva.vainio@metla.fi) (E.J. Vainio).

and Korhonen, 2009). The *H. annosum* complex has a global distribution and includes some of the most destructive forest pathogens in the boreal forest region (Woodward et al., 1998). *Heterobasidion parviporum* Niemelä & Korhonen infects mainly Norway spruce (*Picea abies*), and has a wide distribution in Europe and northern Asia. *H. annosum* (Fr.) Bref. sensu stricto prefers pines (*Pinus* spp.), but infects also other conifers and several deciduous trees in Europe. *Heterobasidion abietinum* Niemelä & Korhonen occurs in central and southern Europe, and infects mainly firs (*Abies* spp.). Also the two North American species, *Heterobasidion irregulare* Orosina & Garbelotto and *Heterobasidion occidentale* Orosina & Garbelotto, have different host tree preferences (*Pinus*, *Juniperus* and *Libocedrus* versus *Abies*, *Sequoiadendron*, *Tsuga*, *Pseudotsuga* and *Picea*; Orosina and Garbelotto, 2010).

The three species, *H. parviporum*, *H. abietinum* and *H. occidentale* are considered to be closely related, and form the 'fir/spruce clade' of the *H. annosum* complex, while *H. annosum* and *H. irregulare* constitute the 'pine clade' (Orosina and Garbelotto, 2010). In North America, *H. irregulare* and *H. occidentale* have been observed to hybridize in nature (Garbelotto et al., 1996), but no natural hybrids between the three European species have been described, although they have been shown to be capable of forming hybrids in the laboratory (Stenlid and Karlsson, 1991). However, *H. occidentale* and *H. annosum* seem to hybridize in Italy (D'Amico et al., 2007; Gonthier et al., 2007), where the North American species was introduced during the Second World War.

Approximately 15% of European and western Asian *Heterobasidion* spp. isolates are infected by dsRNA viruses (Ihrmark, 2001). Thus far, several virus species have been described from *Heterobasidion* species, all of them members of the family *Partitiviridae* (Ihrmark, 2001; Vainio et al., 2010, 2011a, 2011b). Unlike most fungal viruses that require a compatible anastomosis contact for lateral transmission, partitiviruses of *Heterobasidion* spp. are able to transfer horizontally between somatically incompatible strains and even intersterile fungal species (Ihrmark, 2001; Vainio et al., 2010, 2011a, 2011b). They are also capable of spore-mediated dispersal and vegetative spread by cell-to-cell contacts (Ihrmark et al., 2002, 2004).

In this study, we describe the first discovery of a novel virus species designated as *Heterobasidion* RNA virus 6 (HetRV6), found from four species of *Heterobasidion*. The virus was distinct from previously described viruses infecting *Heterobasidion*, and distantly related to the *Curvularia* thermal tolerance virus and the *F. graminearum* virus 4 (Márquez et al., 2007; Yu et al., 2009). A population analysis was conducted to reveal whether there is evidence of co-speciation between the viruses and their *Heterobasidion* hosts at a global scale. We also tested whether the virus is capable of interspecies transmission by hyphal anastomosis. Growth rate tests with isogenic virus-infected and virus-free fungal isolates were carried out on agar plates and Norway spruce

stem pieces to reveal whether the virus affects the growth rate of its host.

## Results

### Sequence statistics and conserved motifs

Complete putative viral polymerase sequences were determined from the *H. abietinum* isolates 04188 and 07052, hosting virus strains designated as HetRV6-ab6 and HetRV6-ab10 (see subsection 'Sequence polymorphism' for virus nomenclature). In addition, primer walking was used to determine a partial sequence comprising the first 1556 nucleotides from the 5'-end of the viral polymerase gene from *H. occidentale* Het6 (host for HetRV6-oc1). The two complete RdRp sequences were both 2050 bp long and contained a single AUG-initiated open reading frame (ORF) of 606 aa (M<sub>r</sub> 69,320 and 69,343 for HetRV6-ab6 and HetRV6-ab10, respectively). The GC-contents of the sequences were 56.8% and 56.2%, respectively. The sequences shared a notably high level of sequence similarity in their 3' UTR regions (100% nucleotide identity over 154 bp), whereas the similarity of the 5' UTRs resembled that of the coding region (96% similarity over 75 bp). The sequence from *H. occidentale* Het6 showed 86% 5' UTR similarity compared to the two sequences from *H. abietinum*.

The 5'- and 3'-terminal UTRs within HetRV6-ab6 and HetRV6-ab10 showed inverted complementarity and could be folded into potential panhandle structures (Fig. S5). In addition, each of the 3' UTRs formed a potentially stable stem-loop structure (Fig. S6), while less inverted complementarity was detected within each of the 5' UTRs of HetRV6-ab6, HetRV6-ab10 and HetRV6-oc1 (Fig. S7).

The conserved motif search implemented in BlastP revealed a conserved region resembling the following RdRp subfamilies in the HetRV6-ab6 polymerase segment: (i) cd01699, aa residues 289–427, E-value 4.76e–06, (ii) pfam 00680, aa residues 303–427, E-value 1.71e–07, (iii) PHA00497, aa residues 293–403, E-value 1.55e–05. The same conserved region occurred also in HetRV6-ab10 and HetRV6-oc1. The conserved RdRp motifs 3–8 of dsRNA viruses of lower eukaryotes, as determined by Bruenn (1993), were located in the sequences as shown in Table 1.

### Phylogenetic affiliation

The three long or complete polymerase sequences (from HetRV6-ab6, HetRV6-ab10 and HetRV6-oc1) were used for phylogenetic inference. Based on BlastP analysis, no sequence matches were found to the previously known viruses of *Heterobasidion* species. The closest matches were with the *Curvularia* thermal tolerance virus, CThTV

**Table 1**

Location of conserved motifs 3–8 as determined by Bruenn (1993) in HetRV6-ab6 and selected dsRNA viruses. The virus name abbreviations are the same as in Table 2 and Fig. 3.

Virus strain	Conserved motifs					
	3 (256–262) <sup>a</sup>	4 (318–327)	5 (379–399)	6 (423–430)	7 (479–483)	8 (505–508)
HetRV6-ab6	TAGRLIF	DAAKFDSSLP	GSTSGHSHNTLMQSICTLVMI	GLGDDQHT	QYL GK	PFDE
CThTV	AVGRLIL	DARKYDAFLD	GTTSGHSHNTLLQSICTLVIG	SLGDDNIT	QYL GK	PFKE
FgV4	PKGRLIL	DAAKFDSSLD	GTTSGHSHNTLLQSICTLVIA	TLGDDNLT	QYL GK	PCEE
HaV	NKLRITW	DWSRFDKRAY	GIPSGLFITQLMDSWYNYVML	VQGDDSI	EVL SY	DL LK
HetRV3-ec1	TKIRVIY	DWSGFDLRSL	SIPSGLFVTFQFLDSHYNLIMI	VQGDDSLI	EVLGY	DMTK
HaV-P	LKVRPVI	DYSRFDQLAP	GVP SGIFMTQILD SFVNL FIF	IQGDDNLV	EVLGY	DVSK
HetRV2-pa1	LKVRPVI	DWSRYDQLLP	GVP SGIFMTQICDSFCNAPLL	IQGDDNVI	EVLGY	DCAK
GaRV-MS1	PKTRLVW	DFSSFDTKVP	GVP SGSWWTQMVDSVYNYILV	VLGDDSAF	KLLGT	DTNE
DdV1	PKTRLVW	DFSAFDSKVP	GVP SGSWWTQIIDS VVNNILI	VLGDDSAF	KLLGT	STDE
BCV3	TKVRGVW	DWSSFDSSVT	GIPSGSYTTSIVGSVYVNRIRI	TQGDDSLI	TFLGR	SLDK
PcV	KKDRITLL	DWADFNEQHS	GLYSGWRGTWINTVNLFCYV	HGGDDIDL	EFLFR	SPTR
Sc L-A (L1)	GKQRAIY	DYDDFNSQHS	TLLSGWRLLTFMNTVNLWAYM	HNGDDVMI	EFLRV	YLSR

<sup>a</sup> Location of the conserved motif in the amino acid sequence of HetRV6-ab6.

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