Heterobasidion dsRNA viruses: diversity, taxonomy and effects

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ABSTRACT

Species of the *Heterobasidion annosum sensu lato (s. l.)* complex are causing root and butt rot of conifers. Mycoviruses are usually cryptic, but some of them may cause hypovirulence (reduced virulence) or mutualistic effects on their fungal hosts. We explored new *Heterobasidion* viruses, and analyzed their taxonomy and effects on their hosts. The viruses were obtained from fungal culture collection of the Natural Resources Institute Finland (Luke) or from newly collected isolates.

This thesis addressed the taxonomy of *Heterobasidion* viruses as well as their transmission, effects on hosts' phenotypes, and distribution. A new dsRNA virus from *H. annosum s.s.,* Heterobasidion RNA virus 6 (HetRV6), was found taxonomically distant from all previously known viruses of *Heterobasidion* spp., but related to the mutualistic Curvularia thermal tolerance virus. Populations of this species exhibited a considerable degree of geographical and host-related differentiation. Virus isolates HetRV6-ab6 and Heterobasidion partitivirus 3 (strain HetPV3-ec1) conferred different and condition-dependent effects on different host strains. Four new partitivirus species, HetPV12, HetPV13, HetPV14 and HetPV15, clustered in a clade within the genus *Alphapartitivirus* that includes also HetPV3 and Helicobasidium mompa partitivirus V70. HetPV13 strains were found to have a high dispersal capacity. A high infection rate by four species of partitiviruses was observed in *H. annosum* in a heavily infected forest. Two of these species were previously unknown (HetPV16 and HetPV20). Three fungal isolates were co-infected by two different partitiviruses (HetPV13-an2 and HetPV7-an1 or HetPV16-an1 and HetPV20-an1), supporting the view that multiple infections are common.

Taken together, the global diversity and prevalence of *Heterobasidion* viruses is considerable, and their transmission may occur between somatically incompatible strains. They may co-infect single host strains, transmit over species borders and confer variable phenotypic effects on their hosts. Further studies are necessary to determine the biocontrol potential of these viruses.

Keywords: Heterobasidion virus, hypovirulence, interspecies virus transmission, HetRV6, phenotypic effect, co-infection.

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"Verily, the only cure for ignorance is to ask questions"- (Prophet Muhammad (PBUH)

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Helsinki, August 2020

Rafiqul Hyder

LIST OF ORIGINAL ARTICLES

This doctoral thesis is based on the following scientific articles and manuscript, which are mentioned numerically. With the kind permissions from publishers, all articles are reprinted here.

I.   Vainio EJ, **Hyder R.,** Aday G., Hansen E., Piri T., Lehtijärvi T. D., Lehtijärvi A., Korhonen K., Hantula J., (2012). Population structure of a novel putative mycovirus infecting the conifer root-rot fungus *Heterobasidion annosum* sensu lato. Virology 422: 366-376. DOI: 10.1016/j.virol.2011.10.032

II.   **Hyder R.,** Pennanen T., Hamberg L., Vainio EJ, Piri T., Hantula J. (2013). Two viruses of *Heterobasidion* confer beneficial, cryptic or detrimental effects to their hosts in different situations. Fungal Ecology 6: 387-396. DOI: 10.1016/j.funeco.2013.05.005

III. Kashif M., **Hyder R.**, De Vega Perez, D, Hantula J, Vainio EJ (2015). Heterobasidion wood decay fungi host diverse and globally distributed viruses related to Helicobasidium mompa partitivirus V70. Virus Research 195: 119-123. DOI: 10.1016/j.virusres.2014.09.002

IV.   **Hyder R.**, Piri T., Hantula J., Nuorteva H., Vainio EJ (2018). Distribution of viruses inhabiting *Heterobasidion annosum* in a pine-dominated forest plot in southern Finland. Microbial Ecology 75:622–630. DOI: 10.1007/s00248-017-1027-6

AUTHOR CONTRIBUTION

Articles	I	II	III	IV
Experimental idea	JH, EV	JH, TPe	EV, JH	TPi, JH, EV
Experimental Design	EV, JH, RH	JH, RH, TPe	EV, KM, RH, JH	EV, TPi, RH, HN
Data collection and experimental work	EV, RH, GA, EH, TPi, TD-L, AL. KK, JH	RH, TPe, EV, JH, TPi	EV, KM, RH, DDVP	TPi, HN, EV
Virus Transmission	EV, RH	RH, EV	EV, KM, RH	
Billet experiment	RH, JH			
Growth experiment including antagonism	EV, RH, JH	TPe, RH		
Analysis of data and results interpretation	EV, RH, GA, EH, TPi, TD-L, AL. KK, JH	RH, TPe, LH, EV, JH, TPi	KM, RH, EV	RH, EV, TPi, JH
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1. Hantula J., Mäkelä S., Xu p., Brusila V., Nuorteva H., Kashif M., **Hyder R.**, Vainio EJ (2020). Multiple virus infection on Heterobasidion sp. Fungal Biology 124: 102-109. DOI: 10.1016/j.funbio.2019.12.004

2. Vainio EJ, Jurvansuu J., **Hyder R.**, Kashif M., Piri T., Tuomivirta T., Poimala A., Xu P., Mäkelä S., Nitisa D., Hantula J.(2018). Heterobasidion partitivirus 13 mediates severe growth debilitation and major alterations in the gene expression of a fungal forest pathogen. Journal of Virology 92(5):JVI.01744-17. DOI: 10.1128/JVI.01744-17

Table of Contents

ABBREVIATIONS

Aa	Amino acid
AM	Arbuscular mycorrhiza
BLAST	Basic local alignment search tool
CHV1	Cryphonectria hypovirus 1
CP	Coat protein
C. parasitica	*Cryphonectria parasitica*
CThTV	*Curvularia* thermal tolerance virus
ECM	Ectomycorrhizal
FgV4	*Fusarium graminearum* virus 4
HetRV	*Heterobasidion* RNA virus (according to old nomenclature)
HetPV	*Heterobasidion* partitivirus (according to new nomenclature, 2016)
H. parviporum	*Heterobasidion parviporum*
H. annosum	*Heterobasidion annosum*
H. ecrustosum	*Heterobasidion ecrustosum*
H. abietinum	*Heterobasidion abietinum*
ISGs	InterSterile Groups
HmPV-V70	Helicobasidium mompa partitivirus V70
HetPV3-ec1	*Heterobasidion* partitivirus 3 from *H.ecrustosum* strain 1 (According to new ICTV classification) [Mentioned in the article 2 as *Heterobasidion* RNA virus 3- ecrustosum 1 (HetRV3)]
HetRV6-ab6	*Heterobasidion* RNA virus 6 from *H. abietinum* strain 6
ICTV	International Committee on Taxonomy of Viruses
P. involotus	*Paxillus involotus*
M. bicolor	*Meliniomyces bicolor*
P. gigantea	*Phlebiopsis gigantea*
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse transcriptase pSPRolymerase chain reaction
R. necatrix	*Rosellinia necatrix*
s.l.	Sensu lato
(+) ssRNA	Positive-sense single stranded RNA
(-) ssRNA	Negative-sense single stranded RNA
ssDNA	Single stranded DNA
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA

1. INTRODUCTION

1.1 *Heterobasidion annosum* species complex

Finland is situated almost entirely in the boreal coniferous forest region, which means that land is covered with tree species like Scots pine (*Pinus sylvestris*, 50.4%), Norway spruce (*Picea abies,*16.2%), two birch species (*Betula pendula* and *B. pubescens* 16.2%) and various other broadleaved tree species (3.5%) (Nygren 2011, Sevola 2007, Forest Resources 2007). This resource serves as a basis for commercial forestry, which is one main industry in Finland. The forest industry is responsible for ca. 20% of the value of the Finnish exports and employs up to 182 000 people (Metsäteollisuus). The forest ecosystem is mostly influenced by annual average temperature (-0.4 to 5.9 °C), duration of sunlight (more or less about 19 to 22 hours during midsummer and about 6 hours during midwinter in southern Finland), and precipitation (~400-650 to ~700-750 mm) (Finnish Annual Weather Forecast). The role of pathogens in forest trees is associated with the economic growth of forest industry. Generally, pathogenic infection by various microbes put the forestry sector in an economic challenge, lowering the quality of timber and thus reducing its value for industrial purposes. The annual damage to Finnish forest owners caused by *Heterobasidion annosum sensu lato* infection is more than 50 million euros (Finnish Ministry of Agriculture and Forestry 2008), and the total losses rise to nearly 800 million euros in Europe (Woodward *et al.* 1998).

The *H. annosum s. l.* complex is widely distributed in Europe and North America (Dai and Korhonen 1999, Dai *et al.* 2003, Ota *et al.* 2006). In Europe, there are three classified species, formerly called as intersterility groups (ISGs), showing specificity towards different hosts (Capretti *et al.* 1990, Korhonen *et al.* 1992, Niemelä and Korhonen 1998, Garbelotto and Gonthier 2013). Indeed, initial classification based on host specificity of the complex was transformed to three ISG types; P (for Scots pine, *Pinus sylvestris*), S (for Norway spruce, *Picea abies*), and F (for Silver fir, *Abies sp.*) (Korhonen 1978, Capretti *et al.* 1990). Nowadays, the species are named as *H. annosum* (formerly called the P-type), *H. parviporum* (formerly called the S-type) and *H. abietinum* (formerly called the F-type) (Niemelä and Korhonen 1998). The North American species *H. irregulare* invades predominantly pines (North American IS group P), whereas *H. occidentale* (North American IS group S) has a wider host range including species of fir, spruce and hemlock (Otrosina and Garbelotto 2010, Garbelotto and Gonthier 2013).

H. annosum s.l. is necrotrophic pathogen that causes root rot for the host, decreasing the value of timber. The *H. annosum* s.l. infects newly cut stump surfaces through spores. After this primary infection is established in stumps, the growth of a fungal mycelium continues as secondary infection via root contacts to other trees (Stenlid and Redfern 1998). *Heterobasidion* spores are airborne, and they land on freshly exposed surfaces of conifer stumps, which are an easy target for colonization if left unprotected after a cutting operation (Rishbeth 1949, 1951). A number of control measures are available to control the *Heterobasidion* infection, including tree species selection (i. e., rotation), stump removal, and chemical or biological stump treatment against spore infection. However, all of these methods have limitations in protecting new infections of *H. annosum* s.l. In Finland, tree species in infected sites may be changed to *Heterobasidion* resistant tree species (e.g. from conifers to broadleaves). However, the soil properties need to allow regeneration with

alternative tree species. In *Heterobasidion*-free sites conifer stump surfaces need to be covered with a control agent. The protection given by stump treatment is not always complete in healthy sites, and stump treatment has no effect on excisting *Heterobasidion* infections. Saprotrophic *Phlebiopsis gigantea* is commonly used as biological competitor to control the infections caused by *H. annosum* and *H. parviporum* in European coniferous trees (Garbelotto and Gonthier 2013, Sierota *et al.* 2015). To prevent stumps from the infection by *Heterobasidion* spores and then further spreading to healthy trees, stump treatment with a chemical (urea) or biological control agent *P. gigantea*, (marketed as PG Suspension® in the UK, PG IBL® in Poland and Rotstop® in Fennoscandia) is recommended (Korhonen *et al.* 1998, Piri *et al.* 1990, Woodward *et al.* 1998, Asiegbu *et al.* 2005, Garbelotto and Gonthier 2013). However, a study demonstrating a natural variation in *H. parviporum* to resist overgrowth by *P. gigantea* strains could be an example of their weak antagonistic performances, suggesting an increasing resistance of *Heterobasidion* spp. towards competitors over time (Samils *et al.* 2008). Furthermore, removing the infected stumps is not an efficient way to remove *Heterobasidion*-infected wood materials from forest, as infected roots remain in the soil and serve as new infection sources (Piri and Hamberg 2015). Most of the *H. annosum* s.l. control methods are preventive, increasing demand for new solutions to minimize the damage by *H. annosum* s.l. in the infected sites. One promising control method of an existing infection is the use of mycoviruses.

Plants are generally associated with complex microbiomes, other than pathogens. Microbes those reside on plant surfaces or within the tissues engage in different types of plant-microbe and microbe-microbe interactions including commensalism, parasitism, mutualism and competition (Campbell 1995). Fungal microbes can be beneficial (mycorrhizae and endophytes), saprophytic (*P. gigantea*) or pathogenic (*Heterobasidion* spp.) to plants. Ectomycorrhizal (ECM) fungi are known to grow in the soil and inhabit roots of all boreal forest trees. Both ECM and arbuscular mycorrhiza (AM) increase the uptake of phosphate (P), other nutrients and water: thus, they can increase the resistance of their host plants against abiotic (e.g., drought, salinity, and heavy metals) and biotic stresses (root pathogens) (Parke *et al.* 1983, Allen and Allen 1986, Duchesne *et al.* 1988, Garcia-Garrido and Ocampo 1988, Bougher *et al.* 1990, Jones *et al.* 1990, Smith and Read 2008). There are a number of ECM species known to restrict the pathogenic root infections (Marx 1972, Perrin 1990), and several ECM fungi were found to be antagonistic to *Heterobasidion* spp. in *in vitro* studies (Napierata-Filipiak and Werner 2000, Mucha *et al.* 2009). Endophytes are known to confer protection directly or indirectly to plants (De lamo and Takken 2020). The direct way includes the interaction with pathogens through mycoparasitism, antibiosis or by competition for nutrients or root niches, and the indirect way includes the induced resistance mechanisms in the host. An endophyte strain of *Fusarium oxysporum* (Fo), Fo47, is reported to reduce disease incidence caused by *Phytophthora capsici* in pepper (Veloso and Díaz 2012), *Pythium ultimum* in cucumber (Benhamou *et al.* 2002) and *Pythium oligandrum* in tomato (Le Floch *et al.* 2009). If using mycoviruses to lower the damage caused by *H. annosum* s.l., the possible virus transmission and effects to other forest fungi (beneficial and saprhophytes) needs to be studied.

1.2 Mycoviruses

1.2.1 Mycoviruses and their taxonomy

Mycoviruses can be defined as viruses that infect and replicate in fungi. Most fungal viruses do not have any extracellular phase in their life cycle. By now mycoviruses have been found in almost all fungal taxa (Pearson *et al.* 2009, Ghabrial 2013, Ghabrial *et al*, 2015).

The genomes of mycoviruses consist of positive-sense single stranded RNA [(+)ssRNA], negative-sense (-)ssRNA, double-stranded RNA (dsRNA), single-stranded DNA (ssDNA) or double-stranded DNA [(dsDNA)] (The ICTV report; http://www.ictvonline.org). Several techniques have been used to detect mycoviruses, including modified CF-11 chromatography (Vainio *et al.* 2010), RT-PCR (Vainio *et al.* 2011a), and high-throughput sequencing (Vainio *et al.* 2015b, Marzano *et al.* 2016).

The International Committee on Taxonomy Viruses (ICTV) classifies mycoviruses into seven dsRNA families *Amalgaviridae, Chrysoviridae, Partitiviridae, Reoviridae, Megabirnaviridae, Totiviridae,* and *Quadriviridae)* and genus *Botybirnaviridae*, eight (+) ssRNA families (*Alphaflexiviridae, Barnaviridae, Botourmiaviridae, Deltaflexiviridae, Endornaviridae, Gammaflexiviridae, Hypoviridae and Narnaviridae*), one (-) ssRNA virus family (Mymonaviridae) and two retrovirus-like elements (*Pseudoviridae* and *Metaviridae*) (ICTV; http://www.ictvonline.org). Members of family *Genomoviridae* have ssDNA genomes. Genus *Botybirnavirus* includes dsRNA viruses. Additionally, a number of other viruses remain unclassified.

In order to characterize the viromes of five fungal plant pathogens including *Colletotrichum truncatum*, *Macrophomina phaseolina*, *Diaporthe longicolla*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum,* Marzano and her group (2016) recently used a metatrancriptomics approach to detect viral sequences. The sequence analysis detected 72 partial or complete genome segments of mycoviruses, 66 of which were previously undescribed. These viruses show similarity to members of families *Partitiviridae, Barnaviridae, Benyviridae, Chrysoviridae, Endornaviridae, Hypoviridae, Narnaviridae, Ophioviridae, Tombusviridae, Totiviridae, Tymoviridae,* and *Virgaviridae*, order *Mononegavirales*, and family *Botourmiaviridae*. They also detected sequences resembling members of the provisionally named virus group "Fusariviridae". Most of the mycovirus species observed in *Heterobasidion* belong to alpha- or betapartitiviruses (Vainio and Hantula 2016, Nibert *et al*. 2014). The classification of partitiviruses was done according to ICTV species demarcation criteria for partitiviruses (Vainio et al. 2018c), which are ≤90% aa-sequence identity in the RdRp and/or ≤80% aa-sequence identity in the CP (Nibert *et al*. 2014).

1.2.2 Mycovirus diversity in Heterobasidion species

Approximately 15-17% of all *Heterobasidion* isolates are infected by dsRNA viruses in Europe and western Asia (Ihrmark 2001, Vainio *et al.* 2011a, Vainio and Hantula 2016). Among the viruses, a high number of species taxonomically belong to *Partitiviridae* (Ihrmark 2001, Vainio *et al.* 2010, 2011a, 2011b, 2012, 2013, 2015a, 2018a), the unassigned CThTV-like virus group (Marquez *et al.* 2007) including HetRV6 and *Narnaviridae* (Vainio 2019). Altogether twenty different species of partitiviruses have been described in *Heterobasidion* spp. (Vainio and Hantula 2016, Vainio *et al.* 2018c). All of them belong to genus *Alphapartitivirus* or *Betapartitivirus* (Vainio *et al.* 2018c).

Heterobasidion partitiviruses are extremely polymorphic (Vainio and Hantula 2016) as they form five clusters in genus *Alphapartitivirus* and two separate clusters in genus *Betapartitivirus* composed of 8 and 12 species, respectively (Vainio *et al.* 2018c). By now partitiviruses from *Heterobasidin* spp. have been found on most continents (Europe, Asia and North America). However, all species of partitiviruses are rare and may constitute less than 30% of dsRNA infections hosted by *Heterobasidion* spp. (Vainio *et al.* 2011a).

1.2.3 General features of partitiviruses

The important features of virus particles of *Partitiviridae* family members include an isometric shape, lack of an envelope, and a bisegmented genome composed of dsRNA of 3-4.8 kbp. Their two genome segments are separately encapsided. According to the ICTV report, members of five genera in this family occur in specific hosts including plants, fungi or protozoa (Vainio *et al*. 2018c). More specific features about the members of the family are presented in Table 1. The presence of one or more additional dsRNA segments is common among members of the family *Partitiviridae*.

1.3 General principles of mycovirus lifestyles

1.3.1 Basic mechanisms for mycovirus transmission

The transmission of mycoviruses with RNA genomes occurs horizontally through hyphal anastomosis and vertically through spores (Ghabrial and Suzuki 2008). Transmission of mycoviruses was found in several fungal species belonging to the same genus. This was true for example in *Cryphonectria* (*C. parasitica* and *C.* sp.), *Sclerotinia* (*S. sclerotiorum* and *S. minor)*, and *Ophistoma* (*O. ulmi* and *O. novo-ulmi*) (Liu *et al.* 2003, Melzer *et al.* 2005).

In vitro studies have shown that the transmission of hypoviruses between strains of *C. parasitica* is possible, but reduced by the phenomenon of vegetative incompatibility governed by six known vegetative incompatibility (vic) loci (Cortesi et al. 2001, Cortesi and Milgroom 1998, Papazova-Anakieva et al. 2008). However, field surveys showed an unclear correlation between the virus incidence and the expected rate of virus transmission (Milgroom and Cortesi 2004, Robin et al. 2010). Brusini and Robin (2013) concluded that vegetative incompatibility barriers are less restricted in the field than in *in vitro* studies. Ding and others (2007) showed the transmission of *C. parasitica* hypovirus during co-culture of virus- positive and virus negative strains of *C. parasitica*. Transmission of these viruses may also occur across incompatible strains (Liu et al. 2003).

In species of *Heterobasidion*, mycoviruses may be transmitted by sexual basidiospores (Ihrmark *et al.* 2004) or asexual conidiospores (Ihrmark *et al.* 2002). Moreover, vegetative transmission of *Heterobasidion* mycoviruses has been frequently noticed between incompatible strains (Ihrmark *et al.* 2002, Vainio 2015a).

Table 1. Characteristics of *Partitiviridae* members (modified from Nibert *et al.* 2014)

Genus	Size differences in genome segments (dsRNA1, dsRNA2, dsRNA3) bp	Size differences in protein length (RdRp/ CP/ RdRp+CP) aa	Host types	Type species	References
Alphapartitivirus	Unique medium size, smaller than betapartitiviruses, combined size (~3750 bp)	Unique medium size, smaller than betapartitiviruses (606/494/ 1097 aa)	Fungi, plants	*White clover cryptic virus 1*	Chiba et al. 2013, Vainio and Hantula 2016
Betapartitivirus	Combined size (~4600 bp)	(717/664/1382 aa)	Fungi, plants	*Atkinsonella hypoxylon virus*	Lesker et al. 2013, Xiao et al. 2014
Gammapartitivirus	Unique combined size but smaller than alpha- and betapartitiviruses (~3300 bp)	Unique combined size (RdRp+CP), smaller than alpha- and betapartitiviruses (537/423/960 aa)	Fungi	*Penicillum stoloniferum virus S*	Oh and Hillman 1995
Deltapartitivirus	Unique combined size but smaller than alpha- and beta-, and gammapartitiviruses (~3164 bp)	Shortest RdRps (477/374/852 aa)	Plants	*Pepper cryptic virus 1*	Sabanadzovic 2011
Cryspovirus	Combined size (~3160 bp)	Shortest CPs (524/319/843 aa)	Protozoa	*Cryptosporidium parvum virus 1*	Nibert et al. 2009

1.3.2 Mycoviruses and their effects on hosts

Because of a latent (cryptic) lifestyle in host cells, mycoviruses have sometimes been considered endogenous or heritable genes (Bruenn 1993, Lemke *et al.* 1979). From the discovery of disease-causing mycoviruses in the cultivated mushroom *Agaricus bisporus* in 1960s, a new era of mycovirology began (Hollings 1962). Mycoviruses may confer

different kinds of effects on host phenotypes, ranging from beneficial (Drinnenberg *et al.* 2013) to symbiotic (Márquez *et al.*, 2007) and detrimental (Choi and Nuss 1992) or even symptomless (Buck 1998). However, due to challenges in experimental infectivity assays, the correlation between a particular mycovirus and the fungal phenotype it confers becomes difficult to infer (McCabe *et al.* 1999). Problems arise especially when mixed infections of several mycoviruses are involved (Howitt *et al.* 2006, Kashif *et al.* 2019).

Typically, during infections mycoviruses do not show any symptoms in the hosts and remain cryptic (Buck 1998). They replicate in the hosts during the infections without affecting the phenotypic properties of the host. Besides, the absence of symptoms in a certain condition does not rule out the possibility of a virus effect in another condition as it was found in *Aspergillus* sp. (Van Diepeningen *et al.* 2006). Generally, mycoviruses that infect *Heterobasidion* spp. remain cryptic. Furthermore, the presence of several mycoviruses in the same host may interfere with each other's actions and thus minimize the hosts' phenotypic alternations, even in cases where individual viruses cause phenotypic effects (Kashif *et al.* 2019).

Hypovirulence can be defined as viral effects causing reduced virulence of the host fungi. There is also a connection between mycovirus causing hypovirulence and fungal phenotypic changes, such as reduced mycelial growth, and sporulation (Hillman *et al.* 2018). The name hypovirus refers to a specific taxonomical group of viruses (members of family *Hypoviridae*). Hypovirulence in fungal strains was first discovered in Italy as resistance factor against chestnut blight cankers (Biraghi 1950). Later in France, hypovirulent strains of *Cryphonectria parasitica* were applied to growing cankers using Grente's method (Grente and Berthelay-Sauret 1978), where mixtures of hypovirulent strains had successfully been applied by chestnut growers in orchards. Several other European countries, e.g., Hungary, Greece, the Slovak Republic and Switzerland, took a similar initiative to convert their local virulent strains with French or local hypovirulent strains to inhibit growing cankers (Juhásová *et al.* 1998, Radócz 1998, Diamandis *et al.* 2014). Due to high diversity in vegetative compatibility, the transmission of Cryphonectria hypovirus 1 (CHV1) has mostly failed to prevent the chestnut blight disease in North America (Choi *et al.* 2012). However, the diseases have been effectively suppressed by using the hypovirulent fungal strains in Europe due to the hypovirus transmission via hyphal anastomosis within the vegetative compatibility group diversity of *C. parasitica* (Milgroom and Cortesi 2004).

Mycoviruses conferring hypovirulence activity have been found in different classes of fungal hosts, for example in *C. parasitica* (Choi and Nuss 1992, Craven *et al.* 1993, Hillman and Suzuki 2004, Nuss 2005), *R. necatrix* (Chiba *et al.* 2009, Kanematsu *et al.* 2010, Xie and Jiang 2014), *S. sclerotiorum* (Xie and Jiang 2014), *Botrytis cinerea* (Castro *et al.* 2003, Xiao *et al.* 2014), *F. graminearum* (Chu *et al.* 2002), *Ophiostoma novo-ulmi* (Deng *et al.* 2003), *Helminthosporium victoriae* (Xie *et al.* 2016, Huang and Ghabrial 1996), *Rhizoctonia solani* (Lakshman *et al.* 1998), *Diaporthe ambigua* (Preisig *et al.* 2000, Smit *et al.* 1996), *Pleurotus ostreatus* (Yu *et al.* 2003), *Agaricus bisporus* (Barton and Holdings 1979), and *Aspergillus* spp. (Diepeningen *et al.* 2008). In a recent study by Vainio *et al.* (2018b), Heterobasidion partitivirus 13 (HetPV13-an1) was shown to cause severe phenotypic alternations in *H. annosum*, including reduced fungal growth *in vitro* as well as in living trees. The virus-caused phenotypic alternations were found not only in the native host but also in a related species *H. parviporum*. The benefit of using homokaryotic *H. parviporum* strains was highlighted in the same study, as they are considered weaker pathogens than heterokaryotic strains of *Heterobasidion* (Korhonen and Piri 1993) and may

allow more efficient virus transmission via mating and anastomosis (Vainio and Hantula 2016).

In contrast to hypovirulence, some mycoviruses are beneficial to their hosts. In the fungi, beneficial effects were found in the 6.0 kbp dsRNA mycovirus in *Nectria radicicola* that up-regulates root-rot associated disease of gingseng (Ahn and Lee 2001). Indirectly, mycoviruses were found to develop a beneficial relationship with hosts such that the competing fungi do not survive in the nutritional niches observed in *Saccharomyces cerevisiae* and other yeasts (Wickner 1996a, Schmitt and Neuhausen 1994), and in *Ustilago maydis* (Koltin 1988). Most notably, killer toxins produced in *S. cerevisiae* against other yeasts confer a competitive benefit to the producer strain, encoded by dsRNA elements called M satellites, which depend for their propagation and maintenance on an L-A helper virus (Drinnenberg *et al.* 2013, Wickner 1996b, Wickner *et al.* 2013). CThTV presents an example of symbiotic mycovirus-fungus effects. This virus confers heat tolerance to a grass plant in the Yellowstone National Park through a three-way symbiosis involving the endophytic fungal host *C. protuberata* (Márquez *et al.* 2007). The grass *Dichanthelium lanuginosum* is able to grow in unnaturally harsh soil conditions where temperature reaches 55°C.

1.4 Co-infection of mycoviruses

Co-infection of mycoviruses is a common phenomenon. It may arise when virus-infected host strains are in close contact. The transmission of virus generally occurs between closely related fungal strains. A fungal strain can be co-infected by two or more viruses. That may affect either one or both viruses, and the association between them does not necessarily depend on the phylogenetical closeness (Kashif *et al.* 2019). Co-infection of mycoviruses was observed also in plant pathogenic fungus *Sclerotinia nivalis* (Wu *et al.* 2016), three distantly related viruses from the *Gremmeniella abietina* type B RNA virus XL (GaBRV-XL) (Tuomivirta *et al.* 2009) and viruses from different families in a single host of *Ustilaginoidea virens* that causes rice false smut in China (Jiang *et al.* 2014). Co-infection of mycoviruses in *Heterobasidion* species was also found in a laboratory study (Kashif *et al.* 2019), as well as in naturally regenerated herb-rich Norway spruce stand and in clonal Norway spruce trees in southern Finland (Vainio *et al.* 2015a, Hantula *et al.* 2020). Thus, the co-infection of mycoviruses is a result of interactions between co-infecting viruses, their relationship to hosts, their transmission levels, and other biological and chemical determinants (Thapa and Roossinck 2019). Search for such factors deepens the understanding of co-infecting viruses and their variable effects between unrelated RNA viruses.

2. OBJECTIVES AND HYPOTHESES

The aim of this study is to improve the understanding of the phylogenetic relationship of *Heterobasidion* mycoviruses, and to explore their possible applicability as biocontrol agents against existing infection of *H. annosum* s.l. Additional aims are to study their effects on fungal hosts' phenotypic properties *in vitro* and/or in woods at various temperatures. Furthermore, this study produced information on the distribution of partitiviruses, their means of transmission, and discrepancies in the phenotypic properties of tested viruses, HetRV6-ab6 and HetRV3-ec1.

2.1 Objectives

The specific objectives of this thesis were:
1. To study the phylogenetics, population structure and host range of a new virus species, HetRV6.
2. To study interspecies transmission through fungal hyphal anastomosis of HetRV6.
3. To compare the positive, negative or cryptic effects on host growth of HetRV6-ab6 and HetRV3-ec1 viruses.
4. To study the effects of mycoviruses on the competitive ability of *Heterobasidion* spp. against other fungi.
5. To characterize globally distributed *Heterobasidion* partitiviruses isolated from *H. annosum, H. parviporum* and *H. irregulare*.
6. To study the distribution of mycoviruses within the host *H. annosum* in the field site.
7. To study the transmission of the mycoviruses through fungal contacts to adjacent fungal strains in a field site.

2.2 Hypotheses

Heterobasidion strains may naturally contain viruses in their cytosol or mitochondria, so the first hypothesis was that the evolution of mycoviruses and their *Heterobasidion* hosts are tightly linked. According to the second hypothesis, interspecies virus transmission through hyphal anastomosis is possible. The third hypothesis related to the degree of virulence of two mycoviruses, HetPV3-ec1 and HetRV6-ab6, stated that the first one possesses a more negative effect and the second one possesses a more positive effect on their fungal hosts' phenotypic properties (i.e. growth rate, competitive ability against other fungi).

We also hypothesized that the virus community in *H. annosum* in a pine forest would be similar to that in *H. parviporum* in a spruce forest. That was addressed via four testable sub-hypotheses which are as follows: (4.1) more than 15% of *H. annosum* strains are infected with mycoviruses in a heavily infected forest site, (4.2) the distribution of mycoviruses is not restricted within the host genotypes, (4.3) the transmission of viruses occur laterally through hyphal contacts to adjacent isolates of plant roots, and (4.4) mycoviruses form multiple infections in *Heterobasidion annosum* strains.

3. MATERIALS AND METHODS

The fungal strains and materials and methods used in this study are summarized in Tables 2 and 3, respectively. Details of the fungal strains and methods will be found in the published articles (I, II, III, and IV) and in their supplemental material. Detection of unknown viruses from fungal mycelia was performed as described in Figure 1.

Table 2. Fungal isolates and mycovirus strains and their relevance

Fungal Isolate	Virus strain ID	Origin	Collectors[a]	Reference
*H. abietinum/*04188	HetPV6-ab6	Turkey	TD, AL	This study (I)
*H. abietinum/*07052	HetRV6-ab10	Austria	GU	
H. occidentale/ Het6	HetRV6-oc1	USA	DG, EH	
*H. abietinum/*07077	HetRV6-ab14	Austria	GU	
*H. parviporum/*95162	HetRV6-pa1	Russia	KK	
*H. abietinum/*04075d	HetRV6-ab3	Turkey	TD, AL	This study (I)
*H. abietinum/*04179	HetRV6-ab4	Turkey	TD, AL	
*H. abietinum/*07047	HetRV6-ab9	Austria	GU	
*H. parviporum/*07057	HetRV6-pa11	Finland	GU	
*H. parviporum/*03107	HetRV6-pa5	Italy	KK	
*H. abietinum/*04188	HetPV6-ab6 free	Turkey	-	This study (I) and (II)
*H. abietinum/*04188	HetPV6-ab6	Turkey	TD, AL	
H. parviporum/ RK5A	virus free	Finland	EV	
H. parviporum/ RK5A[b]	HetPV6-ab6 infected	Finland	-	
H.annosum/ S49-5[b]	virus free	Finland	KK	
H.annosum/ S49-5	HetPV6-ab6 infected	Finland	TP, HN	
*H. ecrustosum/*05166	HetPV3-ec1	China	KK	This study (II)
*H. ecrustosum/*05166	HetPV3-ec1 free	China	-	
H. abietinum/ 00153[b]	virus free	China	-	
H. abietinum/ 00153	HetPV3-ec1 infected	France	CD	
*H. parviporum/*7R15[b]	virus free	Finland	TP	
*H. parviporum/*7R15	HetPV3-ec1 infected	Finland	-	
P. involotus F-CY01[c]	virus free	Finland	-	This study (II)
P. involotus F-YF05[c]	virus free	Finland	-	
P. involotus BOUX[c]	virus free	Finland	-	
M. bicolor R-MF01[c]	virus free	Finland	-	
P. gigantea[d]	virus free	-	-	
H. annosum/ 94221	HetPV12-an1	Poland	PL	This study (III)
H. annosum/ 94233	HetPV13-an1	Poland	PL	
H. annosum/ 05003	HetPV13-an3	Finland	HS, KL	
H. parviporum/ IR41	HetPV13-pa1	Finland	TP	
H. parviporum/ 95122	HetPV15-pa1	Russia	KK	
H. irregulare/ 57002	HetPV14-ir1	USA	JSB	
H. annosum/ S45-8	HetPV13-an2	Finland	TP, HN	This study (III) and (IV)

H. annosum/ S13-2	HetPV13-an1-a	Finland	TP, HN	This study (IV)
*H. annosum/*S45-8	HetPV13-an1-b	Finland	TP, HN	
*H. annosum/*S37-6	HetPV16-an1, HetPV20-an1-b	Finland	TP, HN	
*H. annosum/*K31-6	HetPV16-an1	Finland	TP, HN	
H. annosum/ S35-6	HetPV16-an1, HetPV20-an1-b	Finland	TP, HN	
H. annosum/ S28-5	HetPV16-an1	Finland	TP, HN	
H. annosum/ S43-8	HetPV20-an1-a	Finland	TP, HN	
H. annosum/ 1.11	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ 1.20	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ 1.22	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ 1.30	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ 1.44	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ S32-6	HetPV20-an1-b	Finland	TP, HN	
H. annosum/ T58-6	HetPV20-an1-b	Finland	TP, HN	

[a] AL, A. Lehtijärvi; CD, C. Delatour; DG, D. Goheens; EH, E. Hansen; EV, E. Vainio; GU, G. Unger; HN, H. Nuorteva; HS, H. Schneider; JSB, J.S.Boyce Jr.; KL, K. Lipponen; KK, K. Korhonen; PL, P. Lakomy; TD, T. Doğmus-Lehtijärvi; TP, T. Piri
[b] Virus-free native fungal strains, [c] Symbiotic mycorrhizal fungal strains, [d]Saprotrophic fungi

Table 3. Materials and methods used in this study

MATERIALS AND METHODS	Article
Biology and Microbiology	
Cultivation of fungal cells on artificial media	I, II, III, IV
Cultivation of fungal strains in wood billets	I
Pairing test between virus-donor and virus-recipient fungal strains in malt agar media	I, II, III
Biochemical and Molecular Biology	
DsRNA extraction by CF-11 cellulose affinity chromatography	I, III, IV
Reverse-Transcriptase (RT) Polymerase Chain reaction (PCR)	I, II, III, IV
Complementary DNA (cDNA) synthesis, cloning	I, III, IV
Agarose gel electrophoresis	I, III, IV
Sanger sequencing of cloned inserts	I, III, IV
Single primer amplification technique	I, III, IV
Analytical Programs	
Open reading frames for nucleotide determination by NCBI ORF Finder program	I, III, IV
Molecular Evolutionary Genetics Analysis (MEGA5.1) software version 5 for dendogram construction	I
For dendogram construction of RdRp and CP proteins sequence alignment by MrBayes in Geneious Pro (version 5.5.8 and 5.3)	IV
Statistical programs	
Sequence similarity percentage calculation by Pairwise distance calculation (MEGA 5 and Geneious)	I-IV
Statistical analysis of HetRV6 virus mediated growth rate difference by T-test in Microsoft Excel 2007	I-IV
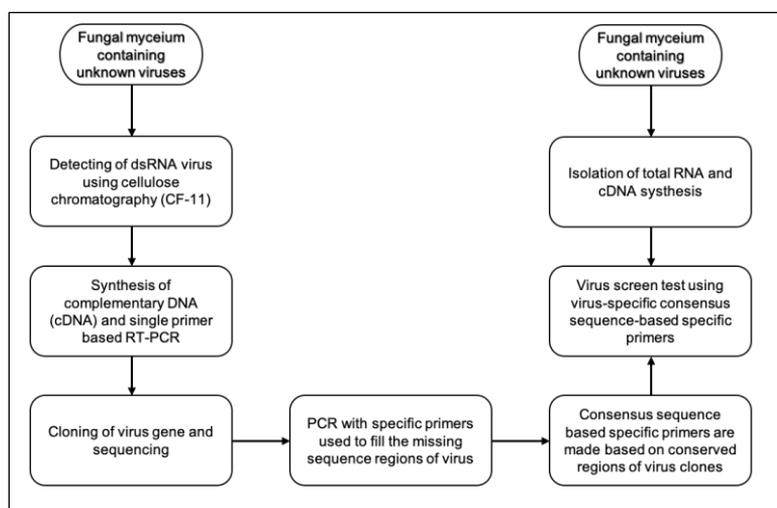
Figure 1. Flowchart of the sample preparation for finding previously known and unknown viruses from fungal mycelia

4. RESULTS AND DISCUSSION

In brief, the results describe (1) the first discovery of a novel virus species HetRV6 and its taxonomical classification, (2) interspecies transmission through fungal hyphal anastomosis of HetRV6 and its phenotypic effects, (3) the positive, negative or cryptic effects of HetRV6-ab6 and HetRV3-ec1 viruses in different *Heterobasidion* hosts, (4) the competitive ability effect of mycoviruses in *Heterobasidion* species against other fungi like mycorrhiza and *P. gigantea*, (5) that phylogenetically *H. mompa* partitivirus V70 is related to *Heterobasidion* partitiviruses isolated from *H. annosum, H. parviporum* and *H. irregulare* (6) the distribution of mycoviruses within the host genotypes in a *Heterobasidion* infected site and (7) the lateral transmission of mycoviruses through fungal contacts to adjacent fungal strains in a *Heterobasidion* infected site in Finland.

Altogether, three Finnish (HetPV7, HetPV16, and HetPV20) (IV), two European (HetPV12 and HetPV13) (III), and two non-European (HetPV14 and HetPV15) (III) strains associated to the *Partitiviridae* family and in addition one phylogenetically yet unassigned (HetRV6) (I and II) virus species were observed. The completely sequenced genomes of the newly found partitiviruses included RdRP and CP genes but only RdRP was observed in HetRV6 (I, III, IV). The phylogenetic analysis of HetRV6 based on partial sequences showed some, but not complete co-evolution between the virus and host fungi (I). Two viruses, HetPV3-ec1 and HetRV6-ab6, were studied for growth rate and competitive ability in three different hosts, but their effects on hosts´ performance were complicated and did not allow their classification as fully beneficial or detrimental, although the overall effect was somewhat negative (II).

4.1 Novel *Heterobasidion* mycoviral strains and their variation within the population community structures

A total of 279 *Heterobasidion* isolates were collected from different locations in Eurasia and North America (I) and tested for the presence of dsRNA. Among them, 35 isolates from three closely related strains (*H. abietinum* 04188 and 07052, and *H. occidentale* Het6) were screened by sequence specific RT-PCR and were found to host a new virus species, named HetRV6. Their cDNA was cloned from three strains (04188, 07052, and Het6) and the viruses were sequenced completely or partially (I). We also observed other viruses in the *Heterobasidion* collection (III), and isolated new viruses from new isolates in a field study (IV). In addition, the sequence data was used for phylogenetic and population genetic analyses to determine the geographic distribution of genetic variants and signals of between-species transmission (I, III). Complete polymerase sequences were determined from two virus strains, HetRV6-ab6 and HetRV6-ab10, whereas the remaining isolates were subjected to partial sequence characterization (I). Genomic composition of these viruses included one segment coding for an RNA dependent RNA polymerase (RdRp), with a size of 2050 bp (I). Taxonomically, the putative virus, assigned as HetRV6, was only distantly related to previously known *Heterobasidion* viruses (I). The unassigned species HetRV6 is responsible for approximately 70% of dsRNA infections in European strains of *Heterobasidion* (I) In our data, it comprised nearly 73% of all fungal isolates analyzed (I) (compared to the total of 15-17% of *Heterobasidion* strains hosting viruses (Ihrmark 2001) in Europe and western Asia. As this virus was not found in *Heterobasidion insulare* s. l., its presence is probably restricted to only *Heterobasidion annosum* s. l. species (I).

Two complete RdRp sequences obtained from HetRV6-ab6 and HetRV6-ab10 (hosts *H. abietinum* 04188 and 07052, respectively), and partial RdRp sequence from HetRV6-oc1 (host *H. occidentale* Het6) viruses revealed their low-level affiliation to CThTV (39-41%), *Fusarium graminerum virus 4* (FgV4) (37%) and very low-level affiliation to other viruses from the family *Partitiviridae* and *Potyviridae* (I). Bayesian analysis and NJ clustering methods also showed these viruses to form a taxonomical cluster together with CThTV and FgV4 in an unassigned virus group (Márquez *et al.* 2007; Yu *et al.* 2009). Viruses belonging to this group have later been found in other ascomycetous and basidiomycetous fungi including endophytic, plant pathogenic and marine fungi (Márquez *et al.* 2007, Nerva *et al.* 2016). BLAST searches showed several closely related viruses resembling CThTV and HetRV6 (Table 4), indicating that similar viruses are geographically widespread and have a wide host range.

HetRV6 also resembles the viruses of the viral family *Amalgaviridae* and a new group 'Unirnaviruses'. Unirnaviruses have non-segmented genomes and include, for example, Beauveria bassiana RNA virus 1 from an entomopathogenic fungus (Kotta-Loizou *et al.* 2015), Beauveria bassiana-like virus from a marine fungus *Penicillium janczewskii* (Nerva *et al.* 2016), Alternaria longipes dsRNA virus 1 from brown rot causing fungus in tobacco (Lin *et al.* 2015), and Ustilaginoidea virens RNA virus M from a rice false smut (Jiang *et al.* 2014).

Plant viruses from *Potyviridae* were clearly dissimilar to HetRV6 due to their huge genomic size and organization (Adams *et al.* 2005), whereas some partitiviruses are similar in size and organization. As HetRV6 is a dsRNA virus related to families *Amalgaviridae* and *Partitiviridae*, it may be provisionally placed in the order *Durnavirales*, class *Duplopiviricetes*, Phylum *Pisuviricota*, Kingdom *Orthornavirae* and Realm *Riboviria*.

The frequencies of HetRV6 viruses among the *Heterobasidion* host species were found uneven based on their partially amplified ca. 700 bp from a total 35 fungal isolates which indicated HetRV6 frequencies of 17.1%, 7.7%, 17.9%, and 14.3% for *H. parviporum, H. annosum, H. abietinum,* and *H. occidentale*, respectively (I). The overall result of the analysis indicated the presence of HetRV6 in a total of 12.5% of analyzed isolates (n = 279) and 72.9% of all dsRNA-positive isolates (n = 35). Interestingly, more than 70% of all *Heterobasidion* viruses found in Finnish forests belong to HetRV6 species (I).

The same ca. 700 bp polymerase sequences encoding 215 amino acid (aa) (645 bp from ~700 bp) were used for the population genetics study (I). All sequences were closely related at the nucleotide and aa level and distinct from other known viruses, and therefore HetRV6 was concluded to be a single novel species (I). Identical protein sequences were found between mycoviruses of *H. abietinum* and *H. parviporum* (HetRV6-ab14; Austria and HetRV6-pa1; Russia); and of *H. abietinum* and *H. parviporum* (HetRV6-ab3, HetRV6-ab4 and HetRV6-ab6; Turkey, were identical to HetRV6-ab9; Austria, HetRV6-pa11; Finland, and HetRV6-pa5; Italy) (III). The two North American strains were clearly different from European isolates (III). Only two virus strains shared identical sequences: HetRV6-ab8 and HetRV6-ab10 from Austrian isolates of *H. abietinum* (I). At the population level, the Eurasian virus strains shared at least 88.5 % and 94.4 % similarity at the nucleotide and protein level, respectively, while the two North American viruses of *H. occidentale* shared 98.1% of nucleotide similarity (I). The similarity between Eurasian and North American viruses varied between 84.3 and 87.9 % at nucleotide and 90.2–93.0% at the protein level, respectively (I). The intercontinental F_{ST} (genetic differentiation among subpopulations) values (0.696–0.762) supported this geographic differentiation (I).

The results obtained supported also some level of host-virus co-evolution as the European virus strains from *H. abietinum*, *H. annosum* and *H. parviporum* had F_{ST} values ranging from 0.230 between viruses of *H. parviporum* and *H. abietinum* to 0.392 between virus strains of *H. parviporum* and *H. annosum*), although the occurrence of highly similar viruses in different host species suggested that also interspecific transmissions occur (I).

4.2 Interspecies virus transmission through fungal hyphal anastomosis and natural distribution

Transmission ability of HetRV6 was characterized on malt agar as dual cultures and the presence of the virus in the new host was confirmed by RT-PCR as described earlier in Vainio *et al.* (2010) (I). The analyses showed evidence of interspecies mycovirus transfer via hyphal contacts to the adjacent heterokaryotic and incompatible *Heterobasidion* species. Interspecies transfer of HetRV6 strains was successful in *in vitro* study from *H. abietinum* (04188) to *H. annosum* (S49-5) and *H. parviporum* (RK5A) via fungal hyphal contacts (I). The genotypes of the donor, recipient and isogenic virus-free control strains were confirmed with mitochondrial and nuclear markers (I).

Interspecies transmission of HetPV3-ec1 virus was also successfully performed on malt agar from *H. ecrustosum* (05166) to *H. parviporum* (7R15) (II) and the presence and stability of the virus in the new host was confirmed by RT-PCR. Transmission on artificial medium was tested via mycelium contacts as shown by Ihrmark *et al.* (2002, 2004) and Vainio *et al.* (2010, 2011b) as well as in natural substrate (II).

The occurrence of phylogenetically closely related viruses in distantly related fungal species and the occurrence of taxonomically distant mycoviruses in the same host fungus were found (III). Similar type of occurrence of virus distribution was found from the inclusion of HmPV-V70 (Osaki *et al.* 2002) within the HetPV3-related virus clade (III). Deng and Boland (2007) showed that interspecies natural transmission of the betapartitivirus *Ceratocystis resinifera* virus 1 to *Ceratocystis polonica*. Similarly, transmission case with partitiviruses was found in *S. sclerotiorum* partitivirus 1 (SsPV1/WF-1) to *S. nivalis* and *Sclerotinia minor* (Xiao *et al.* 2014). Similar strains of *Heterobasidion* virus (HetPV9-pa1) were shared between distantly related fungal species *H. parviporum* and *Megacollybia platyphylla* (Vainio *et al.* 2017). In laboratory conditions, several *Heterobasidion* partitiviruses were transmitted from *H. parviporum* to *H. annosum* and *H. occidentale* via hyphal contacts (Ihrmark *et al.* 2002, 2004) and HetPV3 can be similarly transmitted from *H. ecrustosum* to *H. abietinum* and *H. occidentale* (Vainio *et al.* 2010). Interspecific transmission of partitiviruses is possible also in nature as almost identical strains of HetPV11 were observed in two distinct species, *H. parviporum* and *H. australe*, from Bhutan (Vainio *et al.* 2011a). The natural transmission of mycoviruses is a continuous process, a valuable step for their survival in the changing nature and may be a common phenomenon within *Heterobasidion* species. Evidently, the presence of HetPV13 viruses found in *Heterobasidion* species in two countries (Poland and Finland) separated by nearly 1400 km in distance and with more than 95% identity suggests the viruses have a high dispersal capacity with their hosts (III). The result agrees with the observation of limited diversity, and thus considerable gene flow, between *Heterobasidion* populations in Europe (Stenlid *et al.* 1994).

Table 4. RdRp sequences of Curvularia thermal tolerance virus (CThTV)-like unclassified viruses

Virus name/code	Accession	RdRp size (aa)	Country	Reference	Identity (%) compared to CThTV
Curvularia thermal tolerance virus	YP_001976143	371	USA	Márquez *et al.* 2007	371/371 (100%)
Penicillium aurantiogriseum bipartite virus 1	YP_009182335	614	Italy	Nerva *et al.* 2016	242/349 (69%)
Myriodontium keratinophilum bipartite virus 1	AYP71809	653	Italy	Nerva *et al.* 2019	241/355 (68%)
Cryphonectria parasitica bipartite mycovirus 1	YP_007985675	592	China	Deng *et al.* 2013	182/345 (53%)
Trichoderma harzianum bipartite mycovirus 1	AXU24203	631	China	Liu *et al.* 2019	145/245 (59%)
Heterobasidion RNA virus 6	ADW82833	606	Turkey	(I) This thesis	128/289 (44%)
Sclerotium rolfsii unassigned dsRNA virus	AZF86115	605	China	Zhu *et al.* 2018	140/340 (41%)
Sclerotium hydrophilum virus 1	YP_009273017	624	China	Wang *et al.* 2016	136/334 (41%)
Fusarium graminearum dsRNA mycovirus 4	YP_003288790	712	South Korea	Yu *et al.* 2009	127/332 (38%)
Lactarius tabidus RNA virus 1	AMK47915	680	Finland	Vainio *et al.* 2017	136/344 (40%)
Plasmopara viticola associa-ted partiti-like virus 2	QGZ98414	237	Italy	Chiapello *et al.* 2019	89/170 (52%)
Rhizoctonia solani bipartite-like virus 1	QDW81299	596	Brazil	Picarelli *et al.* 2019	109/270 (40%)

4.3 Phenotypic effect of HetPV3-ec1 and HetRV6-ab6 on *Heterobasidion* species

The majority of the mycorrhizal fungi appeared to be incapable of restricting *Heterobasidion* mycelia in dual cultures (II). However, several Ectomycorrhizal (ECM)

strains *P. involutus*, *M. bicolor* and *Suillus variegatus* were able to restrict the advancement of *Heterobasidion* mycelia by producing a wide inhibition area between the interacting strains (II). As the first two of these ECM strains repeatedly exhibited an inhibition area up to 14 mm between them and *Heterobasidion* strains, except in few cases with hyphal advancement by *Heterobasidion* strains, further studies were performed to test their antagonistic abilities against several *Heterobasidion* species with and without mycoviruses, HetPV3-ec1 or HetRV6-ab6 (II).

The antagonistic interaction of *H. annosum* against saprotrophic fungus *Trichoderma viride* was reported early (Rishbeth 1951) and *P. gigantea* (Rishbeth 1963), but the effect of mycoviruses in *Heterobasidion* hosts on competitive ability was not studied. Therefore, two mycoviruses, HetPV3-ec1 and HetRV6-ab6, with previous information on the virulence in their original native hosts (Vainio *et al*. 2010, Vainio *et al*. 2013), allowed us to formulate a testable hypothesis (II). It was hypothesized that HetPV3-ec1 would affect negatively and HetRV6-ab6 positively their hosts in interaction against mycorrhizal strains and saprotrophic *P. gigantea* (II). Single hyphal-tip cultures of isogenic strains infected and uninfected by HetRV6-ab6 (I) and HetPV3-ec1(II) were used to study the effect of viruses on their hosts growth rate at two different temperatures (6° C and 15° C).

The effects conferred by HetRV3-ec1 and HetRV6-ab6 viruses on the competitive ability of hosts included positive, neutral and negative interactions against other competitors of fungi (II). The significant results of such interactions are shown in the Table 5. Briefly, we had three *Heterobasidion* hosts (*H. parviporum* 7R15, *H. abietinum* 00153 and *H. ecrustosum* 05166) against four different mycorrhizal strains (*P. involutus* F-CY01, *P. involutus* F-YF05, *P. involutus* BOUX and *M. bicolor* R-MF01) (II). Altogether twelve interactions for HetRV3-ec1 virus indicated reducing the hosts's competitive ability in six, increasing in two and no significant effect in four cases (II). The same amount of interactions by HetRV6-ab6 virus in three other *Heterobasidion* hosts (*H. parviporum* RK5A, *H. annosum* S49-5 and *H. abietinum* 04188) against the same mycorrhizal strains showed that HetRV6-ab6 virus reduced hosts competitive ability in five, increasing in three and no effect in four cases (II). In the same study, the experiment against saprotrophic strain *P. gigantea*, HetRV3-ec1 virus in the same three *Heterobasidion* host strains, reduced hosts competitive ability in two cases and increased in one (II). HetRV6-ab6 virus reduced the same host's competitive ability significantly in two out of three cases and had no effect in one against *P. gigantea*. Therefore, considering the indicative and significant results together, it can be concluded that HetRV3-ec1 and HetRV6-ab6 mycoviruses do not uniformly affect *Heterobasidion* host strains competitive ability while interacting against other fungi (mycorrhizas and *P. gigantea*). This indicates that neither of the viruses is harmful or beneficial to the hosts (Table 5). However, overall results indicate that viruses cause slightly more negative than positive effects on the competitive abilities of their hosts (Table 5). Our results were in accordance with a previous study conducted on *Cryphonectria* hypovirus 1 (CHV-1) by Bryner and Rigling (2011), whose work involved the assessment of four CHV-1 subtypes effects on the growth and sporulation of four *C. parasitica* strains at four different temperatures. This indicates that the viruses' uneven effects on hosts were due to temperature-dependent differences in their hosts' physiology. Similar types of variable effects by HetRV3 on its hosts were found in an earlier study indicating the results were strongly dependent on fungal strain, growth medium and incubation temperature (Vainio *et al.* 2010).

The growth rate experiments of isogenic HetRV6-ab6 virus infected and virus-free strains conducted on *H. parviporum* RK5A (I) and *H. annosum* S49-5 (II) resulted in variable outcomes when tested on *in vitro* agar plates (I). The virus had hardly any significant effects on *H. parviporum* as the host growth decreased by up to 6.7% at 6 °C and increased by up to 5.5% at 15 °C (I). On the contrary, the same virus had a significant effect on *H. annosum* strain as the growth rates of the fungal host were increased up to 17.8% at both incubation temperatures, 6 °C and 15 °C (I). In addition, while studied on Norway spruce wood log (billet), the same virus did not produce a significant effect for the *H. parviporum* strain as it increased the growth rate for *H. annosum* by only 5.5% (I). A study conducted by Okada *et al.* (2018), where mycovirus in *Alternaria alternata* displayed two contrasting effects, attenuating host growth and hypervirulence of host plant pathogenicity related to high-titer mycovirus content in between two different hosts, provided a hypothesis for further work i.e., the contrasting behavior of HetRV6 and its hosts might also be explained by the amount of mycoviruses in different mycelia or conditions. Taken together the effects of HetRV6-ab6 on different hosts, either alone or in competitive interactions, allowed us to conclude that the conferred effects of this virus were cryptic or mutualistic depending on incubation temperature and hosts'mycelia (I, II). Thus, the result does not support the view that HetRV6-ab6 virus would have a significant effect on its hosts.

A member of the family *Alphapartitivirus*, HetPV3-ec1, in a new host (*H. parviporum*, 7R15) was used for a growth rate study at two different temperatures (6 °C and 15 °C). Results indicated that virus confers a positive effect to host at low temperatures (II). This was consistent with the earlier study where the same virus also conferred a positive effect to its natural host (*H. ecrustosum* 05166) (Vainio *et al.* 2010). The scenario was different at high temperature where the same virus conferred a negative effect to the host in this study, which was consistent with an earlier study conducted in *H. ecrustosum* and *H. abietinum* but not in *H. parviporum* (Vainio *et al.* 2010). Therefore, the effect of HetPV3-ec1 in different hosts ranged from mutualistic to harmful or cryptic (II). Interestingly, the transcription regulation (Jurvansuu *et al.* 2014) of HetPV3-ec1 genome segments was different from that of other *Heterobasidion* partitivirus species studied, and thus other members of the same species were of interest for further studies. Later, Kashif *et al.* (2019) demonstrated that two co-infecting viruses, HetPV13-an1 and HetPV15-pa1, could provide a stable and negative effect on hosts; whereas, co-infections of HetPV11 and HetPV13-an1 showed variable effects.

4.4 Global distribution of *Heterobasidion* alphapartitiviruses

In this study (III), the complete genome sequences of alphapartitiviruses were determined from four viruses (HetPV12-an1, HetPV13-an1, HetPV13-an2 and HetPV15-pa1) and partially from three viruses (HetPV14-ir1, HetPV13-an3 and HetPV13-pa1). The sequence comparisons showed that there is considerable similarity to previously detected *Heterobasidion* virus strain HetPV3-ec1 (Vainio *et al.* 2010) and *Helicobasidion mompa* partitivirus V70 (HmPV-V70) (Osaki *et al.* 2002). The most common virus species among these four were HetPV13 that appeared as four conspecific strains (HetPV13-an1, HetPV13-an2, HetPV13-an3, and HetPV13-pa1) (III). The polymerase similarity of

Table 5. Effect of viruses on the competitive ability of *Heterobasidion* isolates: direct interactions against mycorrhizal fungi and indirect interaction against *P. gigantea*

Virus	Fungal strains	Direct interaction effect				Indirect interaction effect	Overall reduce effect
		P. inv[1]. F-CY01	*P. inv.* F-YF05	*P. inv.* Boux	*M. bic*[2]. R-MF01	*P. gigantea*	
HetRV3-ec1	*H. parviporum* 7R15	reduce	-	-	reduce	increase	2/5
	H. abietinum 00153	reduce	-	reduce	reduce	reduce	4/5
	H. ecrustusom 05166	increase	increase	reduce	-	reduce	2/5
	Total						8/15
HetRV6-ab6	*H. parviporum* RK5A	increase	-	-	reduce	reduce	2/5
	H. annosum S49-5	reduce	reduce	-	increase	-	2/5
	H. abietinum 04188	-	reduce	reduce	increase	-	2/5
	Total						6/15

[1]*Paxillus involutus,* [2]*Meliniomyces bicolor*

Heterobasidion viruses within-species were up to 97% (at the nucleotide level) in HetPV13 strains isolated from different European locations (III). This confirmed that the alphapartitivirus can be highly dispersed. HetPV13-an1 was originally isolated from *Heterobasdion annosum* strain from Poland, while other three strains were isolated from Finland, indicating that the viruses are far-dispersed. HetPV14 and HetPV15 appeared in single strains of two different hosts (*H. irregulare* and *H. parviporum*), and shared such a low similarity (52.6-67.6% for RdRp and 27.4-28.3% for CP) at the protein or amino acid level (III) that they should be recognized as different species based on ICTV demarcation criteria for partitiviruses (≤90% aa-identity for RdRp and ≤80% aa-identity for CP) (Nibert *et al.* 2014).

In the phylogenetic study, a single strain of HetPV12 virus (III) possessed high CP-sequence similarity (73.7%) with previously described HetPV3-ec1 (Vainio *et al.* 2010) at the protein level, suggesting a relatively close phylogenetic relationship between the two species isolated from two different *Heterobasidion* clusters on different continents (Europe and Asia) (Vainio *et al.* 2011a). Due to a high similarity between two strains isolated from distant origins, it seems that there is no geographical and phylogenetical differentiation among HetPV3 related viruses (III). Our result supports the view of global dispersion of *Heterobasidion* partitiviruses (Vainio *et al.* 2011a). The close association between the conspecific HetPV13 strains and the similarity of HetPV12-an1 and HetPV3-ec1 species was verified by Bayesian RdRp and CP dendrograms and the neighbor-joining dendrogram of the RdRp nucleotide sequences (III). None of the other partitiviruses from

Heterobasidion spp. were closely related to HetPV13 viruses (<43% polymerase sequence identity), but a virus from a different from *H. mompa* of the fungal order, HmPV-V70 order Helicobasidiales, is more closely related (57-67% polymerase sequence identity) (III).

The worldwide distribution of certain mycoviruses in the similar host species might be an evidence of their tightly linked evolutionary pathways. For example, the study of several HetPV13 mycovirus isolates in different *Heterobasidion* hosts from distant locations (~1400 km) showed close genetic similarities (III, Table 1) indicating high dispersal capability. HetPV13-an2 belong to genus *Alphapartitivirus*, observed in *H. annosum* strain S45-8 in Finland (IV), possesses above 97% similarity to HetPV13-an1 in Poland and HetPV13-pa1 in Finland (III). Another partitivirus, HetPV3, rapidly transmitted from *H. ecrustosum* (China) to *H. parviporum* (Finland) (II), *H. annosum* (Finland) and *H. occidentale* (USA) via hyphal contacts in laboratory conditions suggesting that there is a low threshold for interspecific transmission of *Heterobasidion* viruses (Vainio *et al.* 2010).

From our studies of *Heterobasidion* mycoviruses, it seemed that mycoviruses were closely adapted to their hosts (III). This raised a question about possible co-evolution. In case of dsRNA viruses residing in the fungal hosts for long periods of time, being cryptic or active in the host-parasite interactions, several possible scenarios might have happened. Either fungi could have detected them as foreign particles and removed them, or viruses could have controlled the host responses or been ultimately beneficial. In practice, viruses are common among fungi, and thus they have not been removed. Therefore, the viruses may control their hosts or at least avoid host defenses, or live mutually and co-evolve with them in time.

Evidence of co-infection of viruses in a single fungal host was observed throughout our studies (Table 6). Co-infections of both closely (alphapartitiviruses HetPV16-an1 and HetPV20-an1-b) and distantly related (alphapartitivirus HetPV13-an2 and betapartitivirus HetPV7-an1) viruses of the *Partitiviridae* family were found in *H. annosum* (IV). Co-infections of two distantly related alphapartitiviruses (HetPV14-ir1 and a partitivirus resembling *R. necatrix* partitivirus 2) or distantly related virus families (alphapartitivirus

Table 6. List of co-infections of Heterobasidion viruses

Heterobasidion strain	Host code	Infecting virus 1 code (Reference)	Most similar sequence(s) in GenBank	Infecting virus 2 code (Reference)	Most similar sequence(s) in GenBank
H. annosum	S45-8	HetPV7-an1 (IV)	HetPV7-Lt63 (KT733074)	HetPV13-an2 (IV)	HetPV13-an1 (KF963177)
H. annosum	S37-6	HetPV16-an1(IV)	RnPV2[a] (AB569997)	HetPV20-an1(IV)	RnPV2a (AB569997)
H. annosum	S35-6	HetPV16-an1 (IV)	RnPV2[a] (AB569997)	HetPV20-an1(IV)	RnPV2a (AB569997)
H. irregulare	57002	HetPV14-ir1 (III)	*H. mompa* partitivirus V70 (BAC23065)	*R. necatrix* partitivirus 2 (Chiba *et al*, 2013)	*Vicio faba* partitivirus 1 (ABJ99996)
H. annosum	05003	HetPV13-an3 (III)	HetPV13-an2 (KF963179)	HetRV6-an6 (III)	HetRV6-pa19 (KF551893)

[a]RnPV2 = *Rosellinia necatrix* partitivirus 2

HetPV13-an3 and un-assigned HetRV6-an6) were also observed (III). These findings are in accordance with the co-infections of distantly related viruses described in *H. parviporum* (Vainio *et al.* 2015a). In addition to *Heterobasidion* species, co-infections of distantly related partitiviruses (alpha- and betaparitivirus) were also described in *H. mompa* (Osaki *et al*. 2004) and distinct gammapartitiviruses co-infection in *Penicillium stoloniferum* (Kim *et al*. 2005).

4.5 Diversity and spatial distribution of mycoviruses in the *Heterobasidion* hosts and the mode of their dispersal

The distribution of mycoviruses hosted by the *Heterobasidion* isolates of the clones (fungal genotypes) in the ca. 1.5 ha heavily infected forest site in Läyliäinen (southern Finland) was determined (Fig. 2) (IV). We used CF-11 method to find unknown viruses in the study site, and later screened other fungal samples with RT-PCR for the presence of new viruses from disease centers as described in Fig. 1. Virus infections were detected in fungal isolates in only four out of eighteen clones. Some of the infected *H. annosum* isolates among the four clones were found to host variable virus compositions. Furthermore, all isolates observed in the clones were not infected by the same virus. The dsRNA viruses were primarily detected using the CF11 method in 14 isolates (IV). Mycovirus infection of *H. annosum* strains was tested using the RT-PCR method with known virus primers (IV: Table 1), and we developed new ones to detect those were detected by CF11. Finally, additional selective primers specific for HetPV13-an2, HetPV7-an1, HetPV16-an1, HetPV20-an1 and HetPV7+HetPV2 were used for partial genome sequence determination and first four viruses were partly or completely determined (IV). Three of those strains were double infected with either HetPV13-an2 and HetPV7-an1 (strain S45-8) or HetPV16-an1 and HetPV20-an1 (strains S35-6 and S37-6). Initial investigation for viral presence with CF11 chromatography revealed nearly half (nine) of the total virus-positives as RT-PCR detected further virus hosting isolates (IV). However, viruses for two fungal isolates (1.25 and 1.71) could not be detected after the first observations, possibly due to a low titer of viral dsRNA in the mycelium (IV). Based on our combined RT-PCR and sequence information, 29% of the *H. annosum* strains were found to be infected with a virus (IV).

We hypothesized that the distribution of mycoviruses occurs through lateral transmission via *Heterobasidion* anastomoses (IV). Four fungal viruses were detected in several *H. annosum* fungal isolates from pine trees of our study site, designated as HetPV16-an1 (hosts S37-6, S28-5, K31-6, S35-6), HetPV20-an1 (hosts 1.44, 1.11, 1.20, 1.22, 1.30, S32-6, S35-6, S37-6, T58-6), HetPV7-an1 (hosts S143-2, S45-8), and HetPV13-an2 (host S45-8) (IV). The partial genome sequences obtained by cDNA cloning and PCR amplification with specific primers (IV: Table 1) revealed the viruses as members of *Partitiviridae* family. It is notable to mention that during the study, all viral isolates from the fungal isolates of one clone were identical in sequence, whereas conspecific virus strains from different fungal clones (HetPV20-an1 in clones 1 and 9, and HetPV7-an1 in clones 5 and 10) were not identical, which were situated several meters apart (IV). The sequences of conspecific virus strains appeared to differ with a little sequence polymorphism. Under this circumstance, HetPV20-an1 and HetPV7-an1 were not considered to have spread via lateral transmission to other isolates of different clones as their conspecific strains shared RdRp sequences by 98.9% (613 nt) and 100% (296 nt) and CP sequences by 98.3% (354 nt) and 98.4% (674 nt), respectively (IV). The distribution of

these viruses might have occurred in other ways, for example, dispersal of fungal basidiospores, which is consistent with the study by Ihrmark *et al.* (2004). On the other hand, two other viruses, HetPV16-an1 and HetPV13-an2, appeared to be restricted to a single clone (clone 1 and clone 10, respectively) (IV).
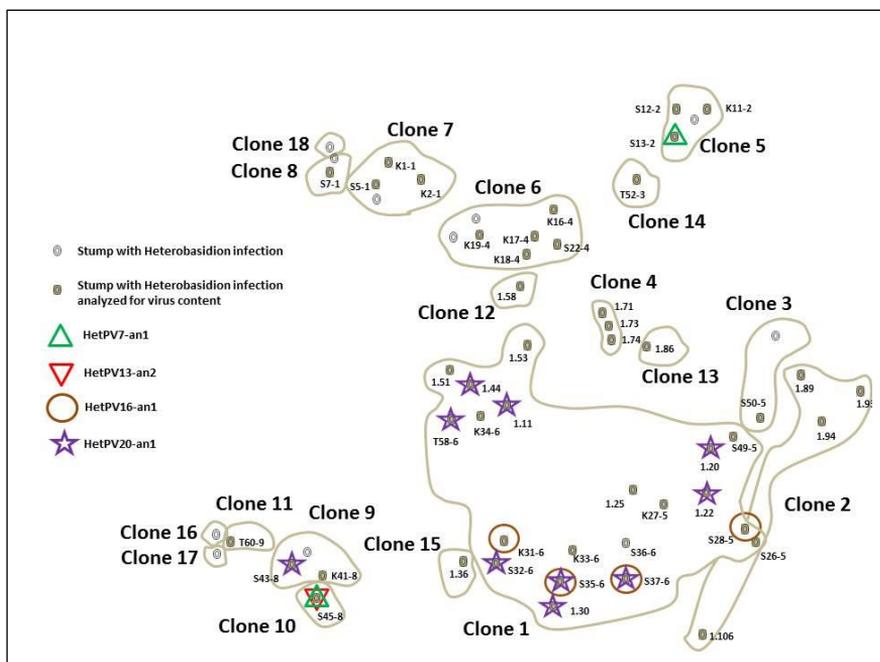

Figure 2. Spatial distribution of *Heterobasidion* clones and viruses infecting them at the study site (IV). Isolates representing the same *Heterobasidion* genotype (clone) are circled and each clone consisting of 1–20 fungal isolates is numbered. The distance between different clones is compressed to show their relative stumps representing different clones are not drawn to scale. The size of the entire study site was ~1.5 ha. (Reprinted from article 4 with permission from Springer Nature)

HetPV7-an1 was found to be conspecific with the previously known betapartitivirus HetPV7-pa1 (Vainio *et al.* 2015a) of *H. parviporum* (97% RdRp identity) (IV). HetPV7-pa1 from *H. parviporum* was highly conserved among local strains at a spruce-dominated forest site with only two point mutations in sequences (Vainio *et al.* 2015a), whereas HetPV7-an1 viruses in the two isolates (S13-2, and S45-8) studied here had three point mutations (IV). The distance between the two locations is ~40 km and the isolates differed by 62 point mutations (IV).

Two novel virus strains, HetPV16-an1 and HetPV20-an1, shared ~70-72% similarity in RdRp nucleotide (nt) sequence with *R. necatrix* partitivirus 2 (RnPV2) and 68% similarity with each other (IV). Family demarcation criteria classified them as new members of *Partitiviridae*. The only partial capsid protein (CP) determined for HetPV20-an1 shared ~40% and ~28% amino acid similarity with that of Rose partitivirus and RnPV2 (IV), respectively.

In this study, the distribution of viruses was not completely explained by lateral transmission through fungal hyphal contacts to the adjacent isolates, but also dispersion of fungal spores might have played a role when viruses were found separated by several meters in distantly inhabiting isolates (Figure 2) (IV). Briefly, the HetPV16-an1 virus was found in four adjacent fungal isolates in the same fungal clone 1, indicating that the isolates infecting stumps were located so closely that the transmission of the virus would probably have moved freely within the mycelium (IV). HetPV13-an2 was detected only in one isolate in clone 10, so there was not any evidence of its transmission to any other isolates (Figure 2) (IV). The HetPV20-an1 virus was also found in nine adjacent isolates of the same clone 1 (Figure 2) (IV) and in one more isolate of the clone occurring several meters away (Figure 2) (IV). HetPV7-an1 was detected in two isolates belonging to different clones that were located several tens of meters away from each other, suggesting that both HetPV7-an1 and HetPV20-an1 transmissions had occurred by other means than through fungal anastomoses (Figure 2) (IV). Overall, the distribution of *Heterobasidion* viruses was uneven among the fungal clones in our plot, which was found also in previous studies in *H. mompa* (Ikeda *et al.* 2005), *R. necatrix* (Yaegashi *et al.* 2013) and the ascomycetous pathogen *C. parasitica* (Shain and Miller 1992, Hoegger *et al.* 2003) as well as *H. parviporum* (Vainio *et al.* 2015a).

Double infection of viruses in a single *Heterobasidion* strain was observed earlier in one of our fungal isolates (94245) harboring both HetRV6-an2 and a partitivirus HetPV1-an3 (formerly, HetRV1-an3) (I). Similar kind of double virus infection was observed in S45-8, where HetPV13-an2 and HetPV7-an1 viruses occurred naturally (IV). Co-existence of different viruses in a single host may also have an evolutionary outcome, as it may allow recombination of viral genomes. HetPV13-an1 virus was capable of reducing host growth *in vitro* as well as in wood (Vainio *et al.* 2018b). An experiment of double infection of both HetPV13-an1 and HetPV15-pa1 resulted in a similar outcome in an experiment by Kashif *et al.* (2019). Furthermore, a study by Vainio *et al.* (2015a) showed that co-infections by distantly related viral species are more stable than those between conspecific strains. This suggests that mutual exclusion might determine mycoviral communities. These hypotheses were not tested here, as the data did not have a temporal design.

The results of our hypotheses tests are described briefly in Table 7.

Table 7. Diversity, taxonomy and effects of viruses as hypotheses and testified results

Hypothesis	Aim	Testified Results
The evolution of mycoviruses and their *Heterobasidion* hosts are tightly linked.	To study the phylo-genetics, population structure and host range of a new virus species, HetRV6.	The hypothesis was partially falsified as strains of HetRV6 did not completely reflect the host species distribution (I).
Interspecies virus transmission through hyphal anastomosis is possible.	To understand if inter-species transmission through fungal hyphal anastomosis is possible by infection experiments between two fungal strains on artificial media.	The statement was supported as the evidence showed mycoviruses to be capable of interspecies transfer among *Heterobasidion* species (I, II, IV).

HetPV3-ec1 possesses more negative effect and the HetRV6-ab6 possesses more positive effect on their fungal hosts' phenotypic properties.	To understand the congruency of positive, negative or cryptic effects of HetRV6-ab6 and HetRV3-ec1 viruses in *Heterobasidion* spp. hosts as determined by growth rate and experiments on the competitive ability against other fungi.	Hypothesis was not completely supported or falsified. Neither of the viruses had only positive nor negative effects. The growth rate by HetPV3-ec1 in *H. parviporum* and by HetRV6-ab6 in *H. parviporum* and *H. annosum* was positive at higher temperature (15°C). Other phenotypic effects (competitive ability against other fungi) conferred by HetPV3-ec1 were mostly negative in *H. parviporum*, *H. abietinum* and *H. ecrustosum*, but positive effects were also observed in few combinations. Likewise, the effects conferred by HetRV6-ab6 were found to be both positive and cryptic in most of the *H. parviporum, H. annosum,* and *H. abietinum* strains (II).
Mycovirus frequency in *H. annosum* is higher (>15%) in heavily infected forest sites than in other locations.	To determine the commonness of mycoviruses in a pine forest site heavily infected by *H. annosum*, and to compare the situation to a forest site heavily infected by *H. parviporum* previously shown to have a high rate of virus infections.	The hypothesis was supported: mycoviruses in *H. annosum* isolates occur frequently (29%) in a forest site heavily infected by *H. annosum* (IV).
Distribution of mycoviruses among *H. annosum* is not restricted within the host genotypes.	To understand the relationship between *Heterobasidion* clones and the distribution of viruses.	The hypothesis was supported because the distribution of mycoviruses was not restricted within a single host in the forest site studied (IV).
Lateral transmission of viruses occurs through fungal hyphal contacts to adjacent clones of *H. annosum.*	To determine how frequently transmissions of the mycoviruses occur in the field through fungal contacts between adjacent strains.	The hypothesis was completely falsified as the transmission of mycoviruses occurred only rarely between adjacent clones (IV).
Mycoviruses form multiple infections in *H. annosum* strains.	To increase knowledge on multiple infections among *Heterobasidion* isolates.	The hypothesis was supported as mycoviruses may occur as multiple infections in a single host depending on host-parasite relationship (I, III, IV).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The study describes virus strains of a novel putative species, HetRV6, that occurs in 12.5% of all the *Heterobasidion* isolates analyzed (n = 279), and in 72.9% of all fungal strains tested dsRNA-positive. Phylogenetically, HetRV6 viruses do not fall into previously assigned families, but they are related rather distantly to the taxonomically unassigned viruses CThTV and FgV4. Phenotypically, the virus is cryptic or possibly neutral or detrimental to its hosts, and it showed geographical and host-dependent differentiation. Horizontal transmission of a virus between sympatric *Heterobasidion* hosts is possible. Thus, the co-evolution of *Heterobasidion* viruses and their hosts is not complete.

HetRV6-ab6 and HetPV3-ec1 showed variable effects (negative, positive or no effect) on their host's growth rate and competitive ability against mycorrhizal and saprotrophic *P. gigantea* strains. Overall, the viruses studied here showed some negative, although only mild, effects on their hosts´ growth rates and competitive abilities, and therefore further screening might result in the discovery of useful biocontrol agents against *Heterobasidion* root rot of conifers. However, the effect of a single host is dependent on environmental and ecological conditions.

The discovery of several closely related HetPV13 strains in *H. parviporum* and in *H. annosum* suggests transmission of viruses between the species. Moreover, a high dispersal capacity of HetPV13 was verified as two identical HetPV13 strains (HetPV13-an1and HetPV13-an2) were found 1400 km apart (Finland and Poland). Phylogenetically, HetPV13-an1 related strains could be interesting for further antagonism studies against other plants root-associated fungi as a recent study (Vainio et al, 2018b) showed successful HetPV13-an1 mediated hypovirulence *in vitro* studies as well as in a plant. Thus, we may consider use of HetPV13-an1 as a potential candidate for bio-control agent to cure *Heterobasidion* infection in plant roots.

The community structure of viruses in *H. annosum* was similar to the previously studied *H. parviporum* in southern Finland. As expected based on earlier studies, the infection rate of mycoviruses in a heavily infected by *H. annosum* pine forest plot was 29%. Four viruses, including two new putative partitiviruses (HetPV16-an1 and HetPV20-an1), were isolated and sequenced from the plot. Conspecific virus pools in different *Heterobasidion* strains were supported by the discovery of HetPV7-an1 from *H. annosum* in our plot, and a closely related virus to HetPV7-pa1 in *H. parviporum* as described earlier. Three cases of co-infections of viruses (HetPV13-an2 and HetPV7-an1 or HetPV16-an1 and HetPV20-an1) in *Heterobasidion* isolates were found indicating that the co-infection of viruses in taxonomically related strains is a common phenomenon.

At this point, our knowledge on mycoviruses, different prospects of them, their roles in hosts and their potential natures are not well known in natural conditions. However, it remains an open issue for future investigation to quest for the ecological role of viruses. One of our studied viruses, HetPV13-an1(III), was found to mediate severe growth debilitations and major changes in the gene expression of hosts in nature as well as in the laboratory study (Vainio *et al.* 2018b). In the phenotypic studies of two viruses (HetRV6-ab6 and HetRV3-ec1), we still do not know whether different isolates of the same fungal species react differently toward infections of a distinct virus strain, because by that we could determine the actual effect (negative or positive) (II). Besides, it would be informative to investigate the level of strain-to-strain variation in the effects of conspecific *Heterobasidion* viruses on their hosts (II). Genetically closely related HetPV13-an1 and HetPV13-an2 viruses would be interesting to study for the role of their genes to understand

mechanisms and genetic background of HetPV13-an1 virulence (IV) (the growth rate of strain S45-8 naturally hosting HetPV13-an2 did not seem to differ significantly from other *Heterobasidion* strains, unpublished data). Additionally, we may also study the effects of HetPV13-an2, HetPV16-an1 and HetPV20-an1viruses in *Heterobasidion* species, other than *H. annosum,* or even in their original hosts in natural conditions (IV). Taken together, *Heterobasidion* viruses and their hosts form a complex net of interactions. It will be interesting to see if they could be of use in restricting the damage caused by the root rots of their hosts.

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