The ISME Journal (2015) 9, 497–507

www.nature.com/ismej

ORIGINAL ARTICLE

Viruses accumulate in aging infection centers of a fungal forest pathogen

This article has been corrected since Advance Online Publication and a corrigendum is also printed in this issue

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Fungal viruses (mycoviruses) with RNA genomes are believed to lack extracellular infective particles. These viruses are transmitted laterally among fungal strains through mycelial anastomoses or vertically *via* their infected spores, but little is known regarding their prevalence and patterns of dispersal under natural conditions. Here, we examined, in detail, the spatial and temporal changes in a mycovirus community and its host fungus *Heterobasidion parviporum*, the most devastating fungal pathogen of conifers in the Boreal forest region. During the 7-year sampling period, viruses accumulated in clonal host individuals as a result of indigenous viruses spreading within and between clones as well as novel strains arriving *via* airborne spores. Viral community changes produced pockets of heterogeneity within large *H. parviporum* clones. The appearance of novel viral infections in aging clones indicated that transient cell-to-cell contacts between *Heterobasidion* strains are likely to occur more frequently than what was inferred from genotypic analyses. Intraspecific variation was low among the three partitivirus species at the study site, whereas the unassigned viral species HetRV6 was highly polymorphic. The accumulation of point mutations during persistent infections resulted in viral diversification, that is, the presence of nearly identical viral sequence variants within single clones. Our results also suggest that co-infections by distantly related viral species are more stable than those between conspecific strains, and mutual exclusion may play a role in determining mycoviral communities.
The ISME Journal (2015) **9,** 497–507; doi:10.1038/ismej.2014.145; published online 15 August 2014

Introduction

Fungal viruses (mycoviruses) occur commonly in all major fungal taxa (Pearson *et al.*, 2009; Ghabrial, 2013). The wide phylogenetic diversity of mycoviruses is reflected by their current classification into seven dsRNA families and five ssRNA families (www.ictvonline.org), and many novel taxa are awaiting description (e.g., Preisig *et al.*, 2000; Ahn and Lee, 2001; Márquez *et al.*, 2007; Hammond *et al.*, 2008; Liu *et al.*, 2009; Yu *et al.*, 2010). Mycoviruses with RNA genomes propagate within the cytoplasm or mitochondria of their hosts and are transmitted laterally between fungal strains through anastomoses. These viruses typically form cryptic (latent), persistent infections, although detrimental as well as mutualistic effects are known (Anagnostakis and Day, 1979; Huang and Ghabrial, 1996; Lakshman *et al.*, 1998; Preisig *et al.*, 2000; Ahn and Lee, 2001; Márquez *et al.*, 2007; Yu *et al.*, 2010). Species of *Hypoviridae* infect the Chestnut blight fungus (*Cryphonectria parasitica*) and reduce its pathogenicity. Consequently, hypoviruses are used as biocontrol agents in Europe (MacDonald and Fulbright, 1991).

Heterobasidion parviporum Niemelä and Korhonen causes white-rot wood decay in Norway spruce (*Picea abies*) and related species (Niemelä and Korhonen, 1998; Dai *et al.*, 2003). This pathogen spreads efficiently *via* basidiospores that infect fresh stump surfaces or butt wounds of living trees and may travel hundreds of kilometers in favorable conditions (Stenlid and Redfern, 1998). A single germinating basidiospore produces a homokaryotic, effectively haploid, primary mycelium; and a compatible pairing between two such mycelia results in the formation of a secondary heterokaryotic mycelium with two different nuclear haplotypes (Korhonen, 1978; Hansen *et al.*, 1993). The mycelium spreads vegetatively *via* root contacts, and single genotypes (clones) may infect dozens of Norway spruce trees and survive for decades. In the case of recent spore-mediated infections, a single conifer stump may harbor several distinct *Heterobasidion* genotypes (Johannesson and Stenlid, 2004). On average, every seventh spruce tree is infected by *H. parviporum* in Southern Finland (Kaarna-Vuorinen, 2000). The disease is currently

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Received 4 December 2013; revised 6 May 2014; accepted 7 July 2014; published online 15 August 2014

controlled by tree species selection, chemical or biological stump treatments and stump removal.

Approximately 15–17% of *Heterobasidion* strains are infected by dsRNA viruses (Ihrmark, 2001; Vainio *et al.*, 2011b). The unassigned species *Heterobasidion* RNA virus 6 (HetRV6) is responsible for approximately 70% of dsRNA infections in European strains of *Heterobasidion* (Vainio *et al.*, 2012) and shares a relatively high polymerase similarity with the *Curvularia* thermal tolerance virus (Márquez *et al.*, 2007). All other *Heterobasidion* viruses described to date are members of *Partitiviridae* (Ihrmark, 2001; Vainio *et al.*, 2010, 2011a, b, 2013b). Many *Heterobasidion* viruses are readily transmitted between incompatible host strains and even distantly related species of *Heterobasidion* in the laboratory and in nature (Ihrmark, 2001; Vainio *et al.*, 2010, 2011a, b). These viruses also spread vertically *via* basidiospores and conidia (Ihrmark *et al.*, 2002, 2004). Most *Heterobasidion* viruses do not cause drastic phenotypic changes in their host, but some may reduce the germination of basidiospores (Ihrmark *et al.*, 2004) or affect the growth of the host fungus (Vainio *et al.*, 2010, 2012). Notably, a single virus strain may have beneficial, commensal or detrimental effects on a single host isolate, depending on environmental and ecological conditions (Hyder *et al.*, 2013). The ecological effects of such complex host–virus relationships can only be understood *via* long-term field studies.

In this study, we deciphered changes in a mycovirus community and its host fungus over a 7-year period in a Norway spruce forest stand suffering from serious *Heterobasidion* infection. We tested the following hypotheses: (i) viruses and fungal host clones are co-distributed, (ii) lateral transmission of viruses occurs between adjacent *Heterobasidion* clones, (iii) viruses accumulate in long-lived clones, (iv) pre-existing viruses influence the probability of other viruses infecting a given host, and (v) accumulated point mutations lead to *in situ* diversification of viruses within a given host.

Materials and methods

Forest site characteristics

The investigated forest site was a naturally regenerated, unthinned, Oxalis-Myrtillus (herb-rich)-type Norway spruce stand in southern Finland (Ruotsinkylä Research Area, 60°22'05'' N, 24°59'53'' E; 875 m^2 area; stand age 43 years). In 2005, 34% of the previous-generation spruce stumps and 32% of standing spruce trees were infected by *Heterobasidion* (for more detailed site characteristics, see Piri and Korhonen, 2008).

Fungal strains and culture conditions

In 2005, Piri and Korhonen surveyed 290 stumps or standing trees for *Heterobasidion* infections and isolated mycelial cultures from 67 of them (Piri and Korhonen, 2008; Figure 1). In 2012, the same 18 stumps and 49 trees (hereafter referred to as 'isolation sources') were resampled by collecting splinters from the stumps or increment cores from the standing trees. Sampling was repeated when no visible decay (i.e., brownish discoloration) was observed at the first sample point. Fungal isolates were assigned individual codes according to the isolation source and year (e.g., isolates retrieved from isolation source 5 in 2005 and 2012 are designated 5-05 and 5-12, respectively). Each of the 290 sample points was assigned a consecutive number by Piri and Korhonen (2008), and we maintained their scheme in this study (small cleaning stumps without *Heterobasidion* infections were omitted from Figure 1).

Airborne *Heterobasidion* spores were trapped using freshly cut, peeled disks of Norway spruce wood that were placed at the study site and exposed in open air for ~24 h. The sampling was repeated twice using 11 disks for each experiment. Cultures were established by collecting multiple conidia from *Heterobasidion* colonies growing on the disks (see Supplementary information for detailed sampling procedure). Sixty *Heterobasidion* isolates were obtained using spore trapping. A schematic of the disk locations is shown in Figure 1b.

The spatial distribution of each *Heterobasidion* clone was determined based on vegetative incompatibility reactions between isolates obtained from different isolation sources, as described earlier (Stenlid, 1985). This method was also used to confirm the identity of a recipient host strain after virus transmission experiments.

To examine whether the congeneric HetPV2 and HetPV7 are able to exist within the same mycelium, we conducted transmission experiments as described by Ihrmark *et al.* (2002) and Vainio *et al.* (2013a). Isolate 18-05 was used as a donor for HetPV2-pa1, and 228-05 was a donor for HetPV7-pa1 in four replicate, dual cultures on 2% malt extract agar plates.

Extraction of dsRNA by CF11 chromatography

The presence of dsRNA in the *H. parviporum* isolates was examined using CF11 cellulose affinity chromatography, as described earlier (Morris and Dodds 1979; Vainio *et al.*, 2010), using approximately 1.5–3.0 g (fresh weight) of fungal mycelia. The CF11 chromatography was repeated if no dsRNA was observed in isolates that yielded viral amplification products *via* RT-PCR.

Molecular cloning and sequencing

Genomic sequences of the new viral species HetPV7 and HetPV9 were determined from isolate 242-05 using the single-primer amplification method of Lambden *et al.* (1992) as modified by Vainio *et al.*

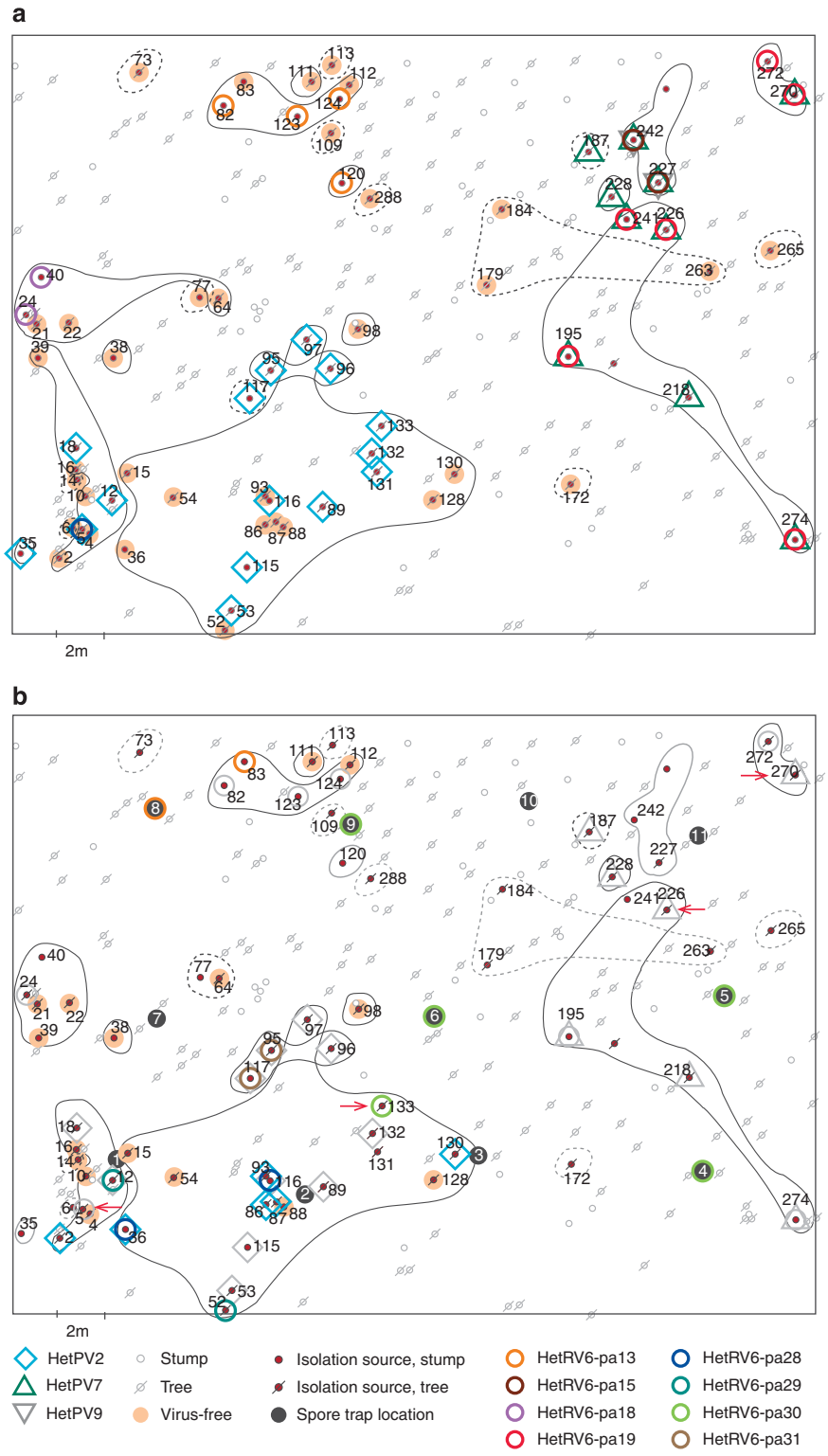

Figure 1 Spatial distribution of viruses and *Heterobasidion* clones at the study site in 2005 (**a**) and 2012 (**b**). The isolation sources refer to individual stumps or trees found to be infected by *H. parviporum* in 2005 and resampled in 2012. Isolation sources infected by the same *Heterobasidion* clone are circled (solid line = heterokaryon; dashed line = homokaryon; black line = viable clone; grey line = clone was not found in 2012). Isolation sources not indicated as virus-infected or virus-free (see symbols) did not yield viable *Heterobasidion* cultures. In 2012 (**b**), viral infections that were already present in 2005 are shown in greyscale, new infections are shown in color, whereas viral losses, when compared to year 2005, are indicated by red arrows.

(2011a). PCR-amplified cDNA products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced at Macrogen Inc. (www.macrogen.com) with an Applied Biosystems 96-capillary ABI 3730xl DNA analyzer (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA). Sequence positions with insufficient coverage were determined using specific primers (Supplementary Table S1). At least three cloned inserts or two overlapping PCR products were analyzed to confirm the identity of each nucleotide.

RT-PCR and partial sequence determination
Reverse transcription was conducted by random priming using total nucleic acid samples, as described earlier (Vainio *et al.*, 2013a). The presence of viruses was examined using selected PCR primers (Supplementary Table S1), and a set of 3–5 primer pairs was used to amplify overlapping fragments covering the entire RdRp coding regions for each viral infection. Any putative mutations were verified by analyzing two independent PCR products from two independent cDNAs. Sequencing PCRs were carried out as described previously (Vainio *et al.*, 2013a) using Dynazyme II DNA polymerase (Thermo Scientific, Vantaa, Finland) and primer-specific amplification conditions (Supplementary Table S1). Sequences determined in this study have been deposited into the NCBI GenBank under the accession numbers listed in Table 1.

DNA extraction and microsatellite markers
We used virus-specific primers and genomic DNA of the host fungus as a template for PCR to investigate whether endogenization of viral sequences into the host genome was possible (Liu *et al.*, 2010; Chiba *et al.*, 2011, see Supplementary Table S2).

Microsatellite markers were determined for representative isolates of each *Heterobasidion* clone to identify putative siblings (Supplementary Table S3). Initially, microsatellite sizes were identified using homokaryotized cultures (i.e., strains obtained by picking individual homokaryotic conidia from heterokaryotic mycelia). Total DNA was isolated from a hyphal mass using a modification of the method described by Vainio *et al.* (1998). Four microsatellite markers were used for the analysis: Ha-ms1, Ha-ms2, Hams6 and Ha-ms10 (Johannesson and Stenlid, 2004). Detailed procedures for homokaryozation, DNA extraction and microsatellite analysis are provided in the supplementary information.

Bioinformatics
Phylogenetic analyses were conducted using Geneious Pro 5.5.8 (Biomatters Ltd., Auckland, New Zealand) and MEGA 5 (Tamura *et al.*, 2011), and Median-Joining Networks were constructed using Network 4.6.1.1. (Fluxus Technology Ltd, Clare, UK). Analyses were conducted with equal weight of characters (10) and a ε-value of zero. Putative recombination events were identified using RDP4 (v.4.16; Martin *et al.*, 2010) with the recombination detection methods RDP, GENECONV, MaxChi, Chimaera, SiScan and 3Seq using default settings for linear sequences and appropriate window sizes.

Results

Spatial distribution of Heterobasidion clones
On the basis of vegetative incompatibility reactions and microsatellite markers, the 67 *H. parviporum* isolates retrieved in 2005 represented 26 individual clones, 12 of which were identified as homokaryons (Figure 1a; Supplementary Table S3). In 2012, new heterokaryotic genotypes were retrieved from stumps 39 and 117, and a new homokaryon was present in tree 64, apparently because of vegetative spread from a neighboring clone (Supplementary Table S3). Several previously infected isolation sources did not yield viable *Heterobasidion* cultures in 2012 (Supplementary Table S2) because of the extensive decay of some stumps or the apparent lack of decayed wood in some standing trees. Most of these standing trees ($N=10$; 83.3%) had been infected by homokaryons in 2005. In 2012, 49 *Heterobasidion* isolates, representing 14 clones, were obtained, and only three isolates were homokaryotic (14-12, 64-12 and 187-12). Note that the majority of trees that had been infected by homokaryons were alive, but two had died by October 2013 (trees 6 and 187). As no thinnings had been conducted at the site; most of the *Heterobasidion* clones are assumed to be long-lived and originate from the previous tree generation and/or stumps generated during the last logging, which took place 43 years ago.

Viral incidence
The analysis of 176 *H. parviporum* isolates revealed 87 viral infections (82 incidences among 116 isolates cultured from wood samples, and 5 incidences among 60 isolates obtained by spore trapping; Supplementary Tables S2 and S4). A total of 47.8% and 67.3% of *Heterobasidion* isolates from wood samples were infected in 2005 and 2012, respectively. At the isolate level, more cases of viruses were gained ($N=14$) than lost ($N=4$) during the sampling period. Most of the *Heterobasidion* isolates retrieved from stumps were infected (2005: 72.2%, 2012: 83.3%), while the frequency was lower (2005: 38.8%, 2012: 62.2%) among isolates from standing trees. If we consider only isolation sources that were infected by *Heterobasidion* in both 2005 and 2012, there were 30 viral incidences in 2005 and 40 in 2012. Despite repeated examination of virus-infected isolates, we did not observe cases of virus loss during storage and subculturing.

Table 1 Viral sequence characteristics

Virus name[a]	*Gene*	*GenBank accession*	*Sequence length*	*Host isolates harboring viral sequence variant*[b]
HetPV2-pa1-a	RdRp	HM565953	2290[c]	[18-05, 18-12]
HetPV2-pa1-a	Capsid	HM565954	2238[c]	—
HetPV2-pa1-b	RdRp	KF551880	2151[d]	[2–12, 5-05, 12-05, 12-12]
HetPV2-pa1-c	RdRp	KF551881	2151[d]	[35-05]; [53-05, 86-12, 87-12, 97-05, 97-12, 115-05, 115-12, 116-05, 116-12, 131-05, 132-05, 133-05]; [95-05, 95-12, 96-05, 96-12, 117-12]; [117-05]
HetPV2-pa1-d	RdRp	KF551884	2151[d]	[89-05, 89-12[e], 93-12, 130-12]
HetPV2-pa1-e	RdRp	KF551882	2151[d]	36-12
HetPV2-pa1-f	RdRp	KF551883	2151[d]	53-12
HetPV2-pa1-g	RdRp	KF551885	2151[d]	132-12
HetPV7-pa1-a	RdRp	JN606091	2297[c] 2157[d]	[187-05]; [228-05, 228-12]; [227-05, 242-05]; [195-05, 195-12, 218-05, 218-12, 226-12, 241-05, 270-05, 270-12, 274-05, 274-12]
HetPV7-pa1-a	Capsid	JN606090	2231[c]	—
HetPV7-pa1-b	RdRp	KF551886	2157[d]	187-12
HetPV7-pa1-c	RdRp	KF551887	2157[d]	226-05
HetPV9-pa1	RdRp	JN606085	2030[c] 1895[f]	[227-05, 242-05]
HetRV6-pa13-a	RdRp	HQ189463	1906[f]	[120-05]; [82-05, 82-12, 83-12, 123-05, 123-12, 124-05, 124-12]
HetRV6-pa13-b	RdRp	KF551888	1930[f]	D8III/1
HetRV6-pa15-a	RdRp	KF551889	2031[f]	242-05
HetRV6-pa15-b	RdRp	HQ189464	1922[f]	227-05
HetRV6-pa18-a	RdRp	JN606086	1881[f]	[24-05, 24-12]
HetRV6-pa18-b	RdRp	KF551890	1846[f]	40-05
HetRV6-pa19-a	RdRp	JN606087	1880[f]	270-05
HetRV6-pa19-b	RdRp	KF551891	1885[f]	[272-05, 272-12]
HetRV6-pa19-c	RdRp	KF551892	1957[f]	226-05
HetRV6-pa19-d	RdRp	JN606089	1946[f]	241-05
HetRV6-pa19-e	RdRp	KF551893	1904[f]	274-05
HetRV6-pa19-f	RdRp	KF551894	1892[f]	274-12
HetRV6-pa19-g	RdRp	JN606088	1913[f]	195-05
HetRV6-pa19-h	RdRp	KF551895	1893[f]	195-12
HetRV6-pa28-a	RdRp	KF551896	1912[f]	[5-05, 5–12]
HetRV6-pa28-b	RdRp	KF551897	1912[f]	[36-12, 116-12]
HetRV6-pa29	RdRp	KF551898	1926[f]	[12-12]; [52-12]
HetRV6-pa30	RdRp	KF551899	1884[f]	[133-12], D4I/5, D5I/4, D6I/4, D9I/3
HetRV6-pa31	RdRp	KF551900	1914[f]	[95-12, 117-12]

[a]Sequence names indicate the viral species (HetPV2, HetPV7, HetPV9 and HetRV6), while suffixes indicate strains (e.g., pa1, pa31), followed by sequence variants (a, b, c etc.).
[b]Isolates of single genets shown in brackets.
[c]Complete sequence including 5′ and 3′ UTRs.
[d]Complete open reading frame (ORF) sequence excluding the forward primer (NPFor) region of 18 bp.
[e]This isolate harbors two sequence variants: HetPV2-pa1-d and HetPV2-pa1-c.
[f]Complete ORF region included.

Virus diversity

Four distinct viral species occurred at the sample plot: three putative members of *Partitiviridae* and the yet-to-be-assigned HetRV6 (Vainio *et al.*, 2012). We found no indications of additional viral strains being present at the study plot, as the dsRNA patterns shown by CF11 chromatography agreed with the RT-PCR results and all dsRNA-positive isolates could be associated with one (or more) of these four viral species by RT-PCR. Viral strains designated as HetPV2-pa1 (*Heterobasidion* partitivirus 2, strain 1 from *H. parviporum*) and HetPV7-pa1 shared 66% and 60% identity in their RdRp and capsid protein sequences, respectively, and could be regarded as congeneric members of the genus *Betapartitivirus* (Nibert *et al.*, 2014). The genome of HetPV2-pa1 (formerly HetRV2-pa1) was characterized by Vainio *et al.* (2011a), and the complete genome sequence of HetPV7-pa1 (*Heterobasidion* partitivirus 7, strain 1 from *H. parviporum*) is presented here. The genome of HetPV7-pa1 consists of an RdRp-encoding segment of 2297 bp, which contains an AUG-initiated open reading frame of 724 aa (M_r 85 348; GC-content 45.6%), and a capsid-encoding segment of 2231 bp, which includes an open reading frame of 654 aa (M_r 73 158; GC-content 50.4%).

HetPV2-pa1 was nearly identical among 32 host isolates (Table 1), with only 0–2 point mutations over 2151 bp (99.9% sequence identity; 9.3×10^{-4} mutations per nt). Notably, one specific mutation (T to A at nt site 691) was characteristic of all HetPV2 infections in the clone residing in isolation source 5, while another viral sequence variant (with an A to

G substitution at nt site 59) was present in isolate 89-05 and two newly infected isolates of the same clone in 2012 (93-12 and 130-12). There were two contradicting cDNAs for isolate 89-12 (A or G at nt site 59), suggesting the presence of two HetPV2 sequence variants. HetPV2 infections in isolates 36, 53 and 132 showed novel point mutations in 2012. All observed mutations resulted in aa substitutions. A Median-Joining Network for HetPV2 sequence variants is presented in Figure 2.

HetPV7-pa1 was highly conserved (Table 1), and only two point mutations were detected among the 17 HetPV7-pa1 sequences of 2157 bp from isolates 187-12 and 226-05 (4.6×10^{-4} mutations per nt; Figure 2). Both substitutions were silent. We have currently found HetPV7 in only one other location, ~40 km from the present study site, and this strain (HetPV7-an1) differed from HetPV7-pa1 by 62 point mutations (Hyder *et al.*, unpublished), which suggests that the single nucleotide microvariations found among variants of HetPV7-pa1 were *in situ* mutations.

The third partitivirus species was designated as HetPV9 (*Heterobasidion* RNA virus 9; Figure 3; Nibert *et al.*, 2014). HetPV9 was found to be identical among isolates 227-05 and 242-05 of the same clone (Figure 1a; Table 1), but no *Heterobasidion* cultures were obtained from these isolation sources in 2012. HetPV9 clusters within the genus *Alphapartitivirus* and shares 68–76% RdRp protein sequence identity with strains of HetPV1 (previously HetRV1; Vainio *et al.*, 2011b; Nibert *et al.*, 2014). The RdRp segment of HetPV9-pa1 (pa1 = strain 1 from *H. parviporum*) was 2030 bp in length and contained an AUG-initiated open reading frame of 620 aa (M_r 72 362; GC-content 47.6%).

HetRV6 was considerably more polymorphic compared with the three partitiviruses, and there were 242 variable sites within an open reading frame region of 1821 bp (the strains shared at least 86.7% identity). To distinguish specific HetRV6 strains from minor sequence variants, we performed a phylogenetic analysis that included published sequences of HetRV6 from distant locations in its global distribution (Vainio *et al.*, 2012). Based on Neighbor-Joining (NJ) analysis (Supplementary Figure S1), eight distinct clusters of HetRV6 sequences (corresponding to viral strains) were recovered from the study site. Notably, HetRV6-pa19 seemed to be more closely related to HetRV6 strains from *H. annosum* than those from *H. parviporum,* suggesting a lateral transfer (i.e., host shift). No recombination events were detected among the HetRV6 sequences.

Most of the eight HetRV6 strains showed minor sequence polymorphisms (1–4 point mutations when compared with the dominant variant; 5.5×10^{-4}-2.2×10^{-3} mutations per nt), yielding 19 HetRV6 sequence variants (Figure 2; Table 1). Spatial and temporal microvariations were observed among HetRV6 infections from single *Heterobasidion* clones. Four ambiguous nucleotide sites were found among the 36 HetRV6 sequences analyzed, which corresponds to a mutation frequency of 6.1×10^{-5}. Specifically, the sequences from isolates 242-05, 195-12 and 272-12 each had a unique nt site showing constant, double base calls (3–4 × sequence coverage), and there were two contradicting cDNAs from isolate 12-12 (nt site 1873). Although this seems to suggest the presence of two molecular variants in a single host isolate, we fixed the character state for the Median-Joining and Neighbor-Joining analyses according to the more commonly occurring nucleotide in the isolation source or clone.

Spatial distribution of viruses

During the 7-year sampling period, there were no new HetPV7 infections and six cases of within-clone dispersal for HetPV2 (isolation sources 2, 36, 86, 87, 93 and 130; Figure 1a and b; Supplementary Figure S2). Eleven isolation sources contained *H. parviporum* strains infected by HetPV2 in both

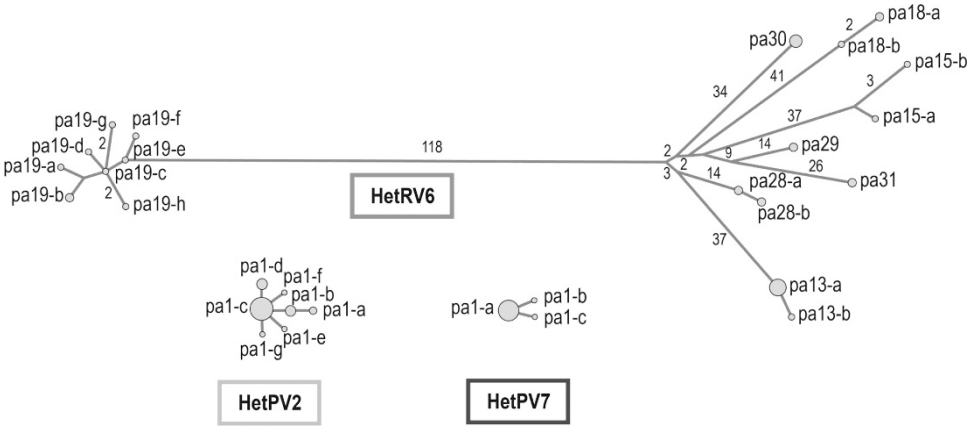

Figure 2 Median-Joining Networks for HetRV6, HetPV2 and HetPV7. Node areas are proportional to the frequency of each detected viral sequence variant. Branch labels indicate the number of base substitutions between sequence variants (only values different from 1 are shown). The lengths of the RdRp sequences were 2151 bp for HetPV2, 2157 for HetPV7 and 1821 for HetRV6.

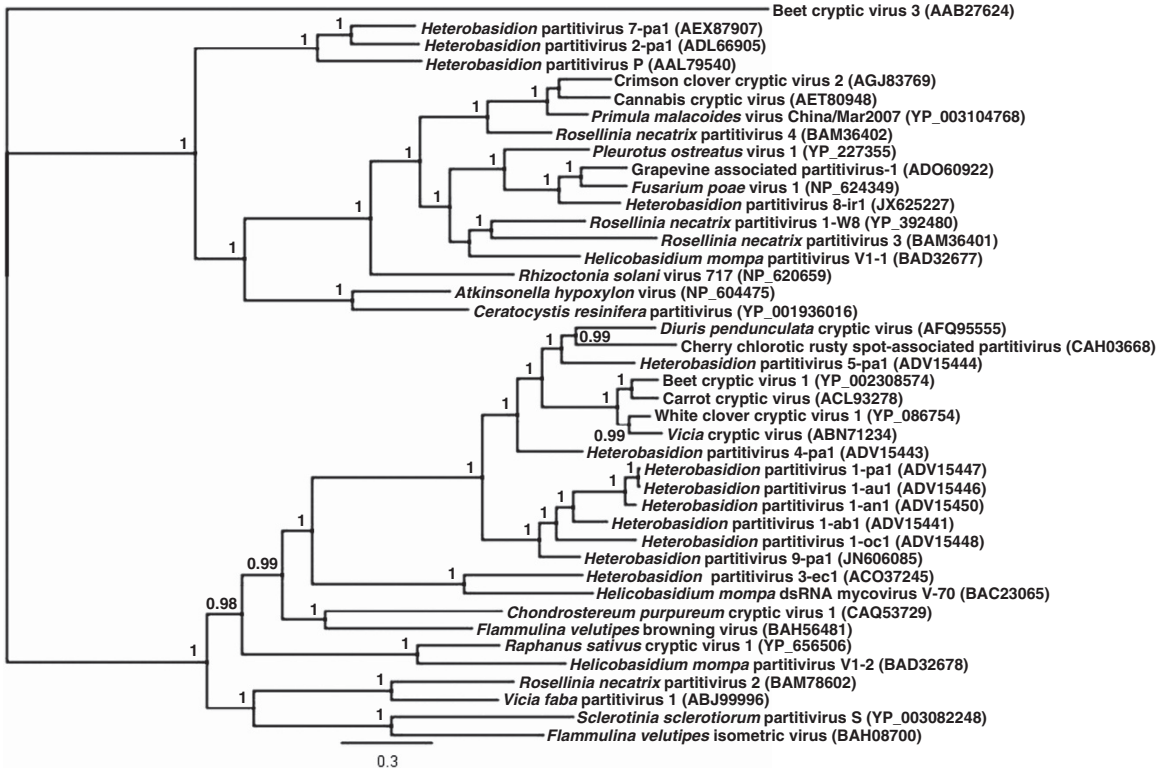

Figure 3 Bayesian dendrogram including HetPV2, HetPV7 and HetPV9 and related species of *Partitiviridae*. The scale bar shows 0.3 aa substitutions per site, and percentages of posterior probabilities over 90% are shown at the branch nodes. The aa sequence alignment was based on complete RdRp sequences, generated using MAFFT version 7.017 and edited manually according to conserved motifs. Phylogenetic clustering was conducted using MrBayes with gamma among-site rate variation and 1.1×10^6 cycles for the MCMC algorithm, with sampling one tree per 200 cycles and discarding 10^5 samples as burn-in. The Blosum matrix was used as the protein evolution model, and Beet cryptic virus 3 was used as an outgroup.

2005 and 2012, and two strains (sources 5 and 133) had lost the virus. In 2012, no cultures were obtained from two isolation sources previously harboring host strains infected by HetPV2. In the case of HetPV7, strains from seven isolation sources were infected by the virus in 2005 and 2012, but no cultures were obtained from three isolation sources in 2012.

Based on microsatellite analysis, most of the five clones inhabited by HetPV2 were not siblings of a single fruitbody (i.e., at least one of the four microsatellite loci did not contain any shared alleles). However, isolate 117-05 seemed to be the homokaryotic constituent of the neighboring heterokaryon, 97-05. The five clones infected by HetPV7 appeared to be more closely related; and isolate 187-05 seemed to be the homokaryotic constituent of the adjacent heterokaryon, 228-05, which, in turn, was a putative sibling of the neighboring isolate, 227-05 (one shared allele at each locus). The clones, including isolates 195-05 and 270-05, were distinct.

The distribution of HetRV6 strains seemed to be more fragmented and temporally variable compared with the partitiviruses (Figure 1b; Supplementary Figure S2). In 2005, three of the five different HetRV6 strains that were found (HetRV6-pa15, HetRV6-pa18 and HetRV6-pa28) occurred solely within single *Heterobasidion* clones, while HetRV6-pa13 and HetRV6-pa19 were found in two neighboring clones. In 2012, three novel HetRV6 strains had appeared at the study site in two clones that were previously devoid of HetRV6. One of the novel strains occurred in two adjacent clones (isolation sources 12 and 52). A new HetRV6 strain was also found from two neighboring isolates of the same clone, 95-12 and 117-12. There was only one case of within-clone virus dispersal for HetRV6: the virus in isolate 82-05 was newly found in neighboring isolate 83 in 2012. Moreover, two new infections seemed to result from inter-clone transmission from isolation source 5 to isolates 36-12 and 116-12 of the neighboring clone. Eight isolation sources contained *H. parviporum* strains infected by HetRV6 in both 2005 and 2012, while strains from two (226, 270) isolation sources had lost the virus in 2012. In 2012, no *Heterobasidion* cultures were obtained from five isolation sources previously harboring HetRV6-infected host strains. None of the clones that shared particular HetRV6 sequence variants (120 and 124 in 2005; 12 and 52 in 2012) were siblings according to microsatellite variation.

HetRV6 was the only viral species that occurred in isolates obtained from spore trapping (8.3% of the 60 established cultures; Supplementary Table S3). Four of these isolates (D4I/5, D5I/4, D6I/4 and D9I/3) were trapped during the first exposure period

(30–31.8.2012), and all were infected by HetRV6-pa30, which was also newly detected in wood isolate 133-12 (i.e., HetRV6 was not detected in 133-05). However, all homokaryotic isolates derived from spores contained microsatellite alleles that were distinct from 133-12 and seemed to have originated from a source outside the study site. Isolate D8III/1, which was infected by HetRV6-pa13, was trapped during the second exposure period (5–6.9.2012). The trapped spore most likely originated from the *Heterobasidion* clone that resided in isolation source 82, which was located ~3 m from the collection disk. The spore-derived D8III/1 and local isolates shared a common microsatellite marker at all four loci and were infected by HetRV6-pa13 sequence variants that differed by only a single point mutation. A *H. parviporum* fruitbody was present in stump 82 in 2012.

Irregular distribution of viruses within Heterobasidion clones

Large clones inhabiting multiple (5–18) isolation sources were all infected by at least one viral species, while clones residing in only one isolation source were mostly virus-free (70.6% in 2005, 71.4% in 2012) and usually homokaryotic. The only homokaryons infected by viruses were 117-05, 187-05 and 187-12, each of which was positioned next to a heterokaryotic clone sharing a common microsatellite haplotype and a common virus strain (HetPV2 or HetPV7, respectively).

Large *Heterobasidion* clones spread over multiple isolation sources were also heterogeneous in their viral content. Isolates of two large clones with 8 and 18 isolation sources showed infection rates of 37.5% and 44.4%, respectively, for HetPV2 in 2005. Similarly, isolates of clones with five to eight isolation sources harboring strains of HetRV6 showed infection rates of 12.5–80% in 2005. Notably, because of novel HetRV6 infections in 2012, each of the two large clones harbored two to three distinct strains of HetRV6, but each virus strain occurred only in one to two isolates, suggesting that the new HetRV6 infections were sporadic (Figure 1b). The possibility of a spontaneous appearance of intact viral RNA from host genomic DNA does not seem likely, as we did not detect viral amplification products using host genomic DNA as a PCR template.

Co-infections by distantly related and congeneric viruses

In 2005, we found natural double infections of HetPV2/HetRV6 (isolate 5) and HetPV7/HetRV6 (isolates 195, 226, 241, 270 and 274) and triple infections of HetRV6/HetPV7/HetPV9 (isolates 227 and 242). In 2012, five new co-infections were observed, in most cases resulting from new HetRV6 infections of *Heterobasidion* strains that already harbored HetPV2 (isolates 12, 95, 116 and 117). Because of viral gains and losses, only two host strains (95 and 274) were infected by the same two viral strains in 2005 and 2012. All viral losses occurred in strains that harbored a second viral species, and in three of four cases, one of the co-infecting viruses was present in both 2005 and 2012.

The congeneric strains HetPV2 and HetPV7 did not naturally form co-infections. To test whether this was due to viral interference, we conducted transmission experiments that produced co-infections of HetPV2 and HetPV7 in isolate 228-05 (all four replicates).

Mutual exclusion of conspecific viral strains

Only one HetRV6 strain was detected from each *Heterobasidion* isolate using HetRV6-specific primers, which suggests the mutual exclusion of closely related HetRV6 strains in the same host. This hypothesis is also supported by the lack of recombination among HetRV6 strains. To reveal putative co-infections, we designed strain-specific primer pairs for HetRV6-pa15 and HetRV6-pa19 (Supplementary Table S5). These viral strains occurred in two adjacent clones, both of which were infected by HetPV7-pa1, suggesting that lateral transfer might have occurred between them. Strain-specific primers and sequence analysis revealed co-infections of HetRV6-pa15 and HetRV6-pa19 in *Heterobasidion* isolates 241-05 and 226-05 (Supplementary Table S5). However, the more rare strain (HetRV6-pa15) occurred in only a few of the tested replicate cDNA samples (12.5–18.2%). All other isolates from the two neighboring clones harbored single strains of HetRV6.

Discussion

In this investigation, we show that *Heterobasidion* isolates at a forest site with a long history of root and butt rot had a viral infection rate of 48–67%, which was considerably higher than the typical 15–17% that was observed in earlier studies (Ihrmark, 2001; Vainio *et al.*, 2012) and suggests that viruses accumulate over time in *Heterobasidion* clones. Our 7-year follow-up work also revealed the appearance of novel viral infections in aging clones. The observation is interesting, as the lifespan of *Heterobasidion* clones is estimated to be considerably shorter (<200 years; Stenlid and Redfern, 1998) than that of some other wood decay fungi that form large clones in the forest (e.g., *Phellinus weirii* and *Armillaria* spp., with a lifespan of more than 1000 years; Dickman and Cook, 1989; Ferguson *et al.*, 2003), and suggests a hypothesis that viruses effectively shorten the lifespan of *Heterobasidion* clones because of their increasing frequency and small but negative overall effect (Hyder *et al.*, 2013).

The distribution of viruses among large *H. parviporum* clones was irregular and suggested that viruses were both acquired and lost during

vegetative growth of the host and/or that the mycelial network comprising a single clone might be partly disconnected. This distribution resembles the uneven distribution of viruses within clones of the pathogenic root rot fungi *Helicobasidium mompa* (Ikeda *et al.*, 2005) and *Rosellinia necatrix* (Yaegashi *et al.*, 2013) and the ascomycetous pathogen *C. parasitica* (Shain and Miller, 1992; Hoegger *et al.*, 2003). We have shown that the partitivirus HetPV4 (formerly HetRV4) was transmitted *via* hyphal contact from an introduced strain of *H. parviporum* into native strains inhabiting Norway spruce stumps (Vainio *et al.*, 2013a). In the present study, partitiviruses seemed to disperse slowly by hyphal contacts between adjacent *Heterobasidion* mycelia, while strains of HetRV6 were able to rapidly infect new clones *via* airborne spores. Notably, the appearance of novel virus strains in aging clones suggests that anastomoses between *Heterobasidion* strains are more frequent than what was inferred from genotype data.

We observed an unexpectedly high frequency of homokaryotic isolates in 2005, although many of them were not detected again in 2012. The occurrence of *H. parviporum* homokaryons in living trees is regarded as a rare phenomenon in Europe, but homokaryons of the closely related *H. occidentale* are known to colonize two or more nearby trees in North America (Garbelotto *et al.*, 1997). Here, we observed several homokaryons, most of which were virus-free, which supports the notion that viruses are relatively rare in basidiospores. However, some of the airborne spores hosted strains of HetRV6, while no partitivirus infections were detected among the spore-trapped isolates. The ability to be transmitted by basidiospores may strongly affect the dispersal of viruses and enables rapid spread into new sites. Ihrmark *et al.* (2004) showed that, depending on the fungal strain, dsRNA viruses are present in 10–84% of basidiospores produced by a virus-infected *Heterobasidion* fruitbody; however, the viral species were not identified. Considering the immense spore deposit from *H. parviporum* fruitbodies (Möykkynen *et al.*, 1997), a continuous spore load could represent a major route of virus transmission, and this is supported by our observation that stumps have a higher viral incidence compared with standing trees, which is expected, as stumps have more exposed and unprotected surfaces that are suitable for spore-derived infections than uninjured trees.

We observed double or triple viral infections in several *Heterobasidion* isolates. Co-infections by distantly related or congeneric mycoviruses have been described (Peever *et al.*, 1997; Lakshman *et al.*, 1998; Preisig *et al.*, 1998; Hong *et al.*, 1999; Ghabrial *et al.*, 2002; Osaki *et al.*, 2004; Park *et al.*, 2005; Tuomivirta and Hantula, 2005; Vainio *et al.*, 2012, 2013a), and the overall similarity of the co-infecting viruses has been relatively low (30–53% at the aa level or <55% at the nt level). Sun *et al.* (2006) have described that the transmission of Mycoreovirus 1 is enhanced by co-infection with Cryphonectria hypovirus 1 in *C. parasitica*. Our results suggested that co-infections by distantly related viral species were more stable than those between conspecific strains, and strains of HetRV6 were able to coexist only transitionally within a single fungal mycelium. If this is a general phenomenon, pre-existing viral infections may affect the dispersal of similar strains and could affect the prevalence of each virus strain within a host population. Partitivirus infections are relatively rare in *Heterobasidion* and occur only in ~5% of isolates in culture collections (Vainio *et al.*, 2011b). Therefore, a single partitivirus strain might potentially spread into several neighboring clones without being restricted through encounters with conspecific strains. In contrast, HetRV6 infections occur in ~12.5% of *Heterobasidion* strains (Vainio *et al.*, 2012), and dispersing viruses are more likely to meet conspecific strains.

The level of intraspecific sequence variation was low for the three partitivirus species, while the HetRV6 population consisted of eight viral strains, three of which showed spatial or temporal microvariations among isolates of single *Heterobasidion* clones. Based on sequence comparisons to previously described viral strains, we deduced that the minor sequence polymorphisms of 1–4 nt were *in situ* mutational events, rather than parallel infections by highly similar strains. Among plants, a single individual may be infected by a swarm of viruses with sequence polymorphisms or so-called 'quasispecies' (Domingo and Holland, 1997), but this phenomenon has been poorly shown in mycoviruses.

Our detailed analysis of a mycovirus community over a 7-year interval has shown that the distribution of *H. parviporum* viruses is spatially influenced by (i) host clonal structure and (ii) lateral transmission of viruses between neighboring clones. We have also shown that (iii) viruses accumulate in aging *Heterobasidion* clones, (iv) the presence of pre-existing viral infections may restrict the dispersal of similar strains, and (v) viral strains acquire point mutations during persistent infections. In conclusion, this study gives unique insights into the dispersal patterns of mycoviruses, which are essential for understanding their biology, their effects on forest mycology and the development of their use as biocontrol agents.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study was financially supported by the Academy of Finland (decision numbers 251193 and 258520) and the Finnish Forest Research Institute. We thank Marja-Leena

Santanen, Juha Puranen, Nieves Lorenzo Gotor, Heidi Ylinenpää, Sonja Sarsila and Suvi Saloranta for technical assistance and Jaana Jurvansuu and Taina Pennanen for commenting on the manuscript.

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